Genetic Analysis of Arabidopsis Nonhost Disease Resistance

Reza Shafiei -Adjbisheh

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Institute of Molecular Plant Sciences
The University of Edinburgh

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Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or other university.

I would like to acknowledge Dr Cui for her invaluable contribution in determining the luciferase activity described in chapter four.

Characterization of *as1* plants was carried out in collaboration with Dr Nurmberg (Reported in chapter 6).

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Thank you all!

Reza

Publications arising from this research

- **Shafiei R**, Hang C, Kang J and Loake GJ (2007) Identification of loci controlling non-host disease resistance in *Arabidopsis* against the leaf rust pathogen *Puccinia triticina*. Molecular Plant Pathology 8(6): 773-784.

- Nurmberg PL, Knox KA, Yun BW, Morris PC, **Shafiei R**, Hudson A and Loake GJ (2007) The developmental selector *AS1* is an evolutionary conserved regulator of the plant immune response. PNAS (In press).

Abbreviations

µg	Microgram
µl	Microlitre
4x*35S*	tetramer of *CaMV 35S* enhancer element
AGT	Appressorium Germ Tube
ANOVA	Analysis Of Variance
AOX	Alternative Oxidase
APF	Appressorium Formation
ARP gene	Orthologs *Asymmetric1*, *Rough Sheath* 2, *Phantastica* genes
AS1	Asymmetric 1 gene
At	*Arabidopsis thaliana*
Avr	Avirulent gene
Bgt	*Blumeria graminis* f.sp. *tritici*
BLAST	Basic Local Alignment Search Tool
Bp	base pair
BZIP	Basic Leucine Zipper
CaM	Calmodulin
CaMV	Cauliflower Mosaic Virus
CC	Coiled-Coil
CE	Cytochalasin E
Cfu	Colony forming units
CGMP	Guanosine 3,5-cyclic monophosphate
Col-0	*Arabidopsis* ecotype
df	Degree of Freedom
DNA	Deoxyribonucleic acid
dpi	Days Post Infection
E.coli	*Escherichia coli*
ET	Ethylene
FEA	Formaldehyde, Ethylene, Acetic acid
FOP	Frequency of Open Stomata
GCD	Guard Cell Death
GFP	Green Fluorescence Protein
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione Redutase

GST	Glutathione S-Transferase
HAF	Haustorium Formation
hpi	Hours Post Infection
HR	Hypersensitive Response
iNOS	Immune Nitric Oxide Synthase
JA	Jasmonic Acid
KB	King's broth media
KNOX genes	*Knotted 1-like homeobox* genes
LRR	Leucine-Rich Repeat
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen Activated Protein Kinase
Me-JA	Methyl-jasmonate
MS	Murashige and Skoog media
MW	Molecular weight standard
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NahG	Salicylate hydroxylase gene
NBS	Nucleotide-Binding Site
NHR	Non-host Disease Resistance
NO	Nitric oxide
NOS	Nitric Oxide Synthase
NR	Nitrate Reductase
$ONOO^-$	Peroxynitrite
PAMP	Pathogen-associated molecular pattern
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDF	plant defensin
PEN genes	Penetration genes
PGT	Primary Germ Tube
PR	Pathogen Related protein
PRR	Pattern recognition receptor
Pst DC 3000	*Pseudomonas syringae* pv *tomato* DC3000
Pst DC 3000	*Pseudomonas syringae* pv *tomato* DC3000
Pst DC 3000 (*avrB*)	*Pseudomonas syringae* pv *tomato* DC3000 carrying *avrB*
Ptr	*Puccinia triticina*
QTL	Quantitative Trait Loci
R	Resistance gene

RNA	Ribonucleic acid
ROI	Reactive Oxygen Intermediate
S.O.V.	Source Of Variation
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SNO	S-nitrosothiol
SNP	Sodium Nitroprusside
SVF	Sub-stomatal Vesicles Fromed
TAIR	The *Arabidopsis* Information Resource
T-DNA	Transfer DNA
TIR	Toll/Interleukin-1 Receptor
TMV	Tobbaco Mosaic Virus

Abstract

Plant immunity against the majority of microbial pathogens is conveyed by a phenomenon termed non-host resistance (NHR) which provides durable protection. In this research, I exploited natural variation in Arabidopsis accessions to reveal the genetic basis of NHR. Significant differences were observed among 79 geographically diverse Arabidopsis accessions in response to the wheat powdery mildew pathogen, *Blumeria graminis* f.sp. *tritici* (*Bgt*) and the wheat leaf rust pathogen *Puccinia triticina* (*Ptr*). In response to *Bgt* genotypes classified into three major classes based on the degree of compatibility, Wc-1 an accession from Germany expressed significantly high frequency of penetration. We also investigated the molecular basis of NHR in Arabidopsis against the wheat leaf rust pathogen, *Puccinia triticina* (*Ptr*). Urediospores germinated with high efficiency and grew randomly over the Arabidopsis leaf surface. However, only 12% of urediospores produced a germ tube that successfully located a stoma and just 0.2% of urediospores went on to produce a haustorium within a penetrated mesophyll cell. Attempted *Ptr* infection induced the production of reactive oxygen intermediates (ROIs), nitric oxide (NO), salicylic acid (SA) and camalexin. The expression of SA, jasmonic acid (JA) and ROI-dependent genes were also detected. A series of well characterized defense-related mutants were challenged with *Ptr*, however, none of these lines exhibited significantly increased susceptibility to this fungus. Our findings also suggest that attempted *Ptr* infection triggers transient stomatal closure in Arabidopsis. We assessed the response of a collection of 79 geographically diverse Arabidopsis accessions to *Ptr*. Wa-1 plants supported a striking increase in *Ptr* sub-stomatal vesicle frequency (SVF) relative to all other tested accessions. Furthermore, SA and camalexin levels became elevated in Wa-1 plants relative to the Col reference line, in response to attempted *Ptr* infection. Additionally, the kinetics of SA-dependent gene expression was expedited in this accession relative to Col plants. To uncover the genetic architecture of NHR against *Ptr*, we assayed the response of the Arabidopsis Landsberg *erecta* (L*er*) x Columbia (Col) recombinant inbred population to this

fungus. Multiple small-to-medium effect quantitative trait loci (QTL) were identified that govern the expression of NHR in Arabidopsis against *Ptr*.

A role for *ASYMMETRIC LEAVES1 (AS1)* in plant immunity has recently been identified. My experiments showed a conserved regulatory function for *NsPHAN*, an orthologue of *AS1* gene in *Nicotiana sylvestris* when challenged with host and nonhost pathogens. This regulatory gene action remained consistent when the *as1* mutant was coupled with key Arabidopsis defence related mutants.

Introduction

The ever increasing demands for food globally, have put pressure on agricultural experts to improve production techniques and introduce new methods to meet future needs. Arable land area only consists of 12% of the total land area (FAOSTAT, 2005), and limitations on developing farmlands due to expansion of warm and arid weather as well as the land demand to be used for other purposes, has led scientists efforts to increase the amount of production per unit of land area. Conventional plant breeding methods have already played an extraordinary role in increasing crop yields. During 1965-1970 wheat production in Pakistan and India almost doubled on the same amount of acreage: this was named the green revolution. The success was achieved through the introduction of dwarf spring wheat cultivars developed by Norman Borlaug (Baenziger *et al.,* 2006; Varshney *et al.,* 2006). Recent advances in plant genomics has revolutionized the capacity of plant breeding by the application of marker-assisted selection (MAS) and the introduction of Genetically Modified (GM) crops. Despite the controversy over growing GM crops, the world wide cultivation of these plants showed an astonishing increase of 11% (equal to 9 million hectares) between 2004-2005, with another five countries, Portugal, France, Czech Republic and Iran (Stone, 2005) joining the pervious 17 countries to grow these crops (James, 2006). However, other biotechnology techniques have made more contributions to crop breeding advances and subsequent increases in production compared to the development of GM crops (Varshney *et al.,* 2006).

Crop yield loss due to pests and pathogens has always been an impasse in agriculture. The estimate of worldwide wheat loss during 2001-2003 was 229 million tons that accounts for 29% of total production, of which 12% is due to pathogen attack (Oerke, 2006). Breeding pathogen resistant crops offers a cost-effective and environmentally friendly strategy to reduce yield losses compared to other plant protection measures, however, the durability and specificity of the resistance remains a major challenge in this field. Plant-pathogen interactions are complex and the durability of resistance is affected by many factors. The

resistance of single genes such as *Sr* (stem rust resistance) *26, Sr2* and *Lr* (leaf rust resistance) *34* in wheat is still effective after prolonged periods of exposure, whereas other single resistance genes lost their effectiveness after a few years in the field (Danial *et al.,* 1995; Schnurbusch *et al.,* 2004; Parlevliet, 2002). Recently, non-host disease resistance (NHR) has been brought to the fore as it conveys both durable and broad-spectrum protection, thus it is of great interest in agriculture. This type of resistance is likely to have a complicated mechanism (Heath, 2000) therefore it is probable that revealing the genetic basis of NHR would not provide a quick applicable tool for engineering resistance in crop plants in the near future.

Plant-Pathogen Interactions

When a pathogen attacks a plant it needs to accomplish several critical stages of pathogenesis such as attachment, penetration, nutrition acquisition and proliferation. On the other hand, plants possess an extensive array of passive and active defence mechanisms, which protect them against a large number of pathogens. Resistance in plants is described as the ability to suppress or retard pathogen activity (Johnson, 1984) which can be attributed to the action of various resistance mechanisms, each functioning at a certain level and specificity (Parlevliet, 2002). These complex and integrated defence mechanisms create the possibility that plants tailor their responses to each type and race of pathogen (Glazebrook, 2005). The earlier level of resistance occurs at the plant's cell wall, which can abolish pathogen penetration through either a pre-formed physical barrier or induced resistance at the penetration site (Yang *et al.,* 1997; Brisson *et al.,* 1994; Brown *et al.,* 1998). The induced resistance at this stage usually comprises cell wall thickening (Brown *et al.,* 1998) along with the accumulation of phenolic compounds and reactive oxygen species (Grant *et al.,* 2000). The outcomes of complex subcellular signalling are pathogen–triggered cell polarization and rearrangement of the cytoskeleton at the penetration site (Yun *et*

al., 2003; Shimada *et al.,* 2006). The crucial role of vesicle trafficking in pathogen penetration demonstrated by pharmacological inhibition of actin microfilament function and the recessive mutation of *PENETRATION* (*PEN*) genes which will be discussed in detail later (Yun *et al.,* 2003; Collins *et al.,* 2003; Lipka *et al.,* 2005).

Pathogens, after successful invasion, undertake different strategies to gain access to plant nutrients. The necrotrophs quickly kill their host after invasion to decompose the plant tissue and to consume the biomass for their own growth requirements. Whereas, biotrophs initiate a close relationship with host cells and maintain a defence incapacity in the plant cell either transiently (hemibiotrophs) or for the duration of the interaction (obligate biotrophs) (Hammond-Kosack and Jones, 1997). In the case of biotrophs, this plant-pathogen interaction is established through the haustorium, a specialized fungal structure. Haustoria primarily function to uptake nutrients from plant cells (Hahn and Mendgen, 2001; Voegele and Mendgen, 2003), reassemble their cytoskeleton (Riemann *et al.,* 2002) and influence their host plant's metabolism and defence responses (Voegele and Mendgen, 2003). The most economically important haustorium-forming pathogens still cannot complete their life cycle *in vitro*. The remarkable features of biotrophic fungi are their highly developed infection structures; limited activity for the secretion of enzymes, especially lytic enzymes; carbohydrate rich and protein-containing interfacial layers that separate fungal and plasma membranes; long-term suppression of host defence; and haustoria for nutrient absorption (Mendgen and Hahn, 2002).

Penetration can occur either through high physical pressure, like rice blast (*Magnaporthe grisea*), which is a hemibiotroph or by secreting digestive enzymes as in grey mould (*Botrytis cinerea*), a necrotroph pathogen (Doss, 1999; White and Traquair, 2006; Doss *et al.,* 2003). In case of necrotrophs, pathogen penetration is followed by the secretion of phytotoxic molecules and lytic enzymes to kill the cell. Some necrotrophs such as *B. cinerea* trigger the host oxidative burst (Deighton *et al.,* 1999) and possibly the hypersensitive response

(HR) to assist with effective invasion (Govrin and Levine, 2000). Soon after cell death, enzymes such as endopolygalacturonases, pectin methylesterases, cellulases and endo-β-1,4-xylanase which belongs to family 11 of glycosyl hydrolases, convert plant biomass to be used by the fungus (Brito *et al.,* 2006; Valette-Collet *et al.,* 2003). The wide range of hosts for necrotrophic pathogens demonstrates their ability to neutralize plant phytotoxins through detoxification (Daoubi *et al.,* 2005; Farooq *et al.,* 2002) and is an indication of less effective resistance through a plant toxic metabolomics approach. However, other strategies for induction of resistance could be more efficient. For instance, inhibition of *Botrytis* endopolygalacturonase protein by overexpression of polygalacturonase inhibiting proteins in Arabidopsis reduced disease symptoms significantly (Ferrari *et al.,* 2003).

Recognition of a pathogen is pivotal to the activation of plant defence systems (Glazebrook, 2001) which is characterized by the subsequent accumulation of defence signalling molecules, reactive oxygen species (ROS) (Grant and Loake, 2000) and nitric oxide (NO) (Delledonne *et al.,* 1998). This leads to the activation of the two major mechanisms that control the plant defence system; salicylic acid (SA)-dependent signalling and jasmonic acid (JA)- and /or ethylene (ET)-dependent signalling. These pathways are independent, however, in some cases such as wounding their responses may overlap (Glazebrook, 2005). In this context, *R* gene-mediated resistance has been very well studied, while the defense mechanisms which are activated in a non-specific fashion remain to be categorized (Takken *et al.,* 2006; Klarzynski and Fritig, 2001).

Pathogen Effectors

Effectors are a set of pathogen molecules introduced into the host to manipulate the function of the plant cell for their own benefit (Torto *et al.,* 2003). Thus, effectors can either antagonise host resistance or trigger the defence response. A

pool of effectors is usually secreted by a pathogen which functions as a 'tool kit' in host plant. Effectors may comprise virulence factors like AVR proteins (Mackey and McFall, 2006; Jones and Dangl, 2006).

Bacterial pathogens have three different protein-secretion pathways to deliver effectors; type II secretion system (T2SS) for soft rotting pathogens like *Erwinia* spp, Type III (T3SS) for the bacterial flagellum and Type IV (T4SS) essential for pathogenesis of *Agrobacterium tumefaciens*. Large numbers of bacterial pathogens deliver effectors through the type III secretion system through plant cell walls to translocate proteins across the host plasma membrane into the host cytoplasm (Abramovitch and Martin, 2005). There are also large numbers of bacteria that employ multiple protein secretion systems like *Erwinia* and *Xanthomonas* (Preston *et al.,* 2005). Delivery of effectors in fungal pathogens is categorized based on their target sites. Those effectors that are secreted into the plant extracellular space are named apoplastic effectors, where they interact with extracellular targets and surface receptors. The other group is cytoplasmic effectors which are translocated inside the plant cell through specialized structures such as haustoria or infection vesicles that invaginate inside living host cells (Kamoun, 2006). An example of apoplastic effector is AVR2 from *Cladosporium fulvum*. This protein binds to the tomato extracellular cysteine protease RCR3 and the extracellular domain of tomato R protein Cf-2 sense the interaction which promotes an HR (Rooney, 2005). Whereas, AvrL567 from flax rust, *Melampsora lini* is secreted through haustoria and detected in the cytoplasm (Dodds *et al.,* 2004).

There is also great diversity of effectors between and within plant pathogen species (Kamoun, 2006). Thus, pathogen take advantages of their highly polymorphic effectors to suppress resistance by sequence diversity (Kamoun, 2006; Catanzariti *et al.,* 2006; Dodds *et al.,* 2006). Proteins encoded by the *AvrL56* in flax rust are recognized by three R proteins of flax, however, 12 sequential variants for this gene have already been identified in rust flax strains (Dodds *et al.,* 2006). Another example is the effector gene *Arabidopsis thaliana*

Recognized1 (*ATR1*) of *Hyaloperonospora parasitica*, which is highly polymorphic although it has a conserved RXLR motif (Rehmany *et al.*, 2005). RXLR is a conserved motif within different oomycete genomes and within avirulence factors (Rehmany *et al.*, 2005). For instance, *ATR13* from *Phytophthora ramorum* (Allen *et al.*, 2004), *Avr3a* from *Phytophthora infestans* (Armstrong *et al.*, 2005) and *Avr1b* from *Phytophthora sojae* (Jiang *et al.*, 2006) all have a RXLR motif. Nevertheless, pathogens are still able to overcome unspecific rejection mechanisms (Jones and Takemoto, 2004).

Pathogen Associated-Molecular Patterns

Should pathogens successfully overcome the preformed resistance barriers of the host then an active resistance mechanism is induced to combat the pathogen. The first layer of recognition takes place through identifying a highly conserved and sensitive part of the pathogen termed pathogen-associated molecular patterns (PAMP) (Jones and Dangl, 2006). PAMPs are vital for pathogens and it is indispensable for the pathogens to release them (Parker, 2003). These are known as general elicitors that are characteristic of a whole class of pathogens. The function of these patterns are vital for pathogens with a low mutation rate and they are not found in higher Eukaryotes (Gomez-Gomez, 2004). In plants, PAMPs are sensed by transmembrane pattern recognition receptors (PRRs) (Zipfel *et al.*, 2004), which results in PAMP-triggered immunity (PTI) and contributes to the immunity of plants at a very early stage (Jones and Dangl, 2006). In animals, PTI is mediated by a family of conserved transmembrane Toll-like receptors (TLR). TLR extracellular domains contain LRR and its intracellular domain has TIR domain (Beutler and Rehli, 2002). PTI induces relatively weak resistance responses (Jones and Dangl, 2006), however, PAMPs seem to be very important in activation of immunity system and controlling the pathogen development (Zipfel *et al.*, 2006).

There is limited knowledge about PAMP receptors in plants, however, the information is fast growing. One of the PAMP receptors in Arabidopsis is *Flagellin Sensing* (*FLS2*). *FLS2* encodes a receptor-like protein kinase (RLK), carrying a transmembrane domain, an intracellular serine/threonine kinase domain and an extracellular LRR domain (Gomez-Gomez and Boller, 2000). This PAMP receptor triggers resistance through a Mitogen-Activated Protein (MAP) kinase signalling cascade (Asai *et al.,* 2002). The impact of *FLS2* is the recognition of a 22-amino-acid peptide (flg22) from a highly conserved fragment of the bacterial flagellin domain (Zipfel *et al.,* 2004). Flagellin is required for bacterial motility and viability (Gomez-Gomez and Boller, 2002). Remarkably, in less than one hour, treatment of Arabidopsis with a synthetic flg22 triggered a rapid transcriptional induction of 966 genes. On the other hand, bacterial growth was decreased dramatically on the plants that were pre-treated with flg22. Arabidopsis *fls2* mutants expressed more susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) when bacteria were sprayed on plants. While following infiltration of inocula into the leaves, wild-type and mutant plants remained similar, suggesting a role for *FLS2* at earlier stages of pathogen attack (Zipfel *et al.,* 2004). Despite the conserved structure of PAMPs and PRRs, both of them are subject to natural selection. For instance, Ws-0 is non-responsive to flg22 due to the point mutation in its *FLS2* (Zipfel *et al.,* 2004; Jones and Dangl, 2006).

Non-host Disease Resistance

Non-host resistance (NHR) describes the situation that an entire plant species is immune to a specific parasite (Heath, 2001), a phenomenon that is not yet well understood. Alternatively, host resistance is commonly controlled by parasite-specific resistance (*R*) genes, but the role of these genes in non-host resistance is controversial and probably varies with the plant–pathogen combination (Heath, 2001). NHR in contrast to gene-for-gene resistance, is durable and effective

against a broad spectrum of pathogens, the characteristics that are much valuable for agriculture (Ellis, 2006). While NHR effectively prevents an invading pathogen to complete its life cycle, the resistance phenotype would be categorised in two classes (Mysore and Ryu, 2004). The first type of NHR response has no visible symptoms due to the success of defence system before the pathogen is able to penetrate the plant. On the other hand, type II NHR produces visible symptoms, alternative to the disease phenotype mainly in form of hypersensitive response (HR). In this context, the pathogen has successfully overcome barriers of penetration, but surrounded by the subsequent induced defence mechanisms (Mysore and Ryu, 2004).

The first layer of resistance that contributes to NHR is the preformed barriers. Among of these are saponins, preformed plant secondary metabolites with antifungal activity. Saponins constitutively produced by some plants species (Hostettmann and Marston, 1995; Osbourn, 1996). It was shown that *saponin-deficient* (*sad*) mutants of oat (*Avena strigosa*) are compromised in resistance to the following host and non-host pathogens; *Gaeumannomyces graminis* var. *tritici* isolate T5, *Fusarium culmorum*, *F. avenaceum* and *G. graminis* var *avenae* (Papadopoulou *et al.,* 1999).

Although the physical structure of plant cells may contribute to non-host resistance, this rarely can completely account for the lack of successful pathogen infection (Yun *et al.,* 2003; Nishimura *et al.,* 2003). For instance, synchronized interference in actin microfilaments (a key component of the cytoskeleton) polymerization and *EDS1* gene function in Arabidopsis severely compromised non-host resistance against wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*), however, sporulation was a rare phenomena (Yun *et al.,* 2003). Likewise in a host plant resistance, loss of callose synthase in *pmr4* (*powdery mildew resistance 4*) mutant mounted an alternative defence pathway, depended on salicylic acid which resulted in enhanced level of resistance rather than susceptibility in the mutant plant (Nishimura *et al.,* 2003).

The reality that some responses of plants to non-host pathogens are similar to the responses to host pathogens raised the assumption that NHR could possibly share some host-pathogen signalling pathways and activate similar defence mechanisms (Heath, 1981; Thordal-Christensen, 2003). Responses such as callose deposition (Heath and Skalamera, 1997), ROS accumulation (Mellersh *et al.,* 2002), adhesion of the plasma membrane to the cell wall (van't Slot *et al.,* 2003) and the involvement of the *suppressor of G2 allele of SKP1* (*SGT1*) gene which is required for *R* gene mediated resistance (Peart *et al.,* 2002) are also expressed against non-host pathogens. However, the difference between these defence systems may lie in their timing and localization, as NHR is normally effective at the earliest time point and is extracellular while host resistance is an intracellular response which occurs at later stages (Abramovitch *et al.,* 2006). Nevertheless, it seems that some signalling events affect the immune system globally. For example, glutathione *S*-nitrosoglutathione reductase (GSNOR) regulates *S*-nitrosylation in cell and a mutation in *Arabidopsis thaliana GSNOR* (*AtGSNOR*) *1* gene caused enhanced level of cellular S-nitrosothiols (SNOs) which subsequently compromised *R* gene-mediated defence, basal resistance and non-host responses (Feechan *et al.,* 2005). In contrast to wild type Arabidopsis, plants carrying *Atgsnor1-3*, a loss-of-function mutation in *AtGSNOR1*, supported increased growth of non-host pathogens like *Bgt* and *P. syringae* pv *phaseolicola*. Thus, indicating a requirement for *AtGSNOR1* in NHR (Feechan *et al.,* 2005).

Recently, several genes have been identified that compromise NHR. Mutations in *PENETRATION (PEN)1, PEN2* or *PEN3* resulted in increased penetration rates of *Blumeria graminis* f.sp. *hordei* (*Bgh*) spores into Arabidopsis cells six, seven and five folds respectively. However, pathogens ceased to develop further, due to other resistance reactions particularly the hypersensitivity response (Collins *et al.,* 2003; Lipka *et al.,* 2005; Stein *et al.,* 2006). *PEN1* encodes a plasma membrane resident syntaxin, a member of the SNARE (soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptor) super family. SNARE proteins family direct membrane fusion during all steps of trafficking, which restricts pathogen invasion into the plant cell wall (Collins *et al.,* 2003). In *Colletotrichum*-Arabidopsis

interaction, PEN1 is involved indirectly in the reduction of penetration, although it was not functionally essential to prevent penetration (Shimada *et al.,* 2006). *PEN2* encodes a family 1 glycosyl hydrolase (F1GHs) (Lipka *et al.,* 2005). Cell wall modification is one of the extensive range of activities that this enzyme family are involved in (Xu *et al.,* 2004) and the PEN2 Green Fluorescent Protein (GFP) fusion construct showed a link between *pen2* and mobile vesicle-like bodies (Fig 1.1). Apparently, *pen1* and *pen2* are in different pathways as their double mutant showed an 11 fold increase of penetration (Lipka *et al.,* 2005). *PEN3* encodes the putative ATP binding cassette (ABC) transporter, PLEIOTROPIC DRUG RESISTANCE8 (PDR8) and the higher penetration observed on *pen3* mutants resulted from the loss of function of PDR8. Unlike the other *PEN* genes, *PEN3* contributes to defences both at the cell wall and intracellularly (Stein *et al.,* 2006).

The *nonhost* (*nho*)*1* mutant was first identified in Arabidopsis (Lu *et al.,* 2001). The *NHO1* gene encodes a glycerol kinase. In *nho1* plants NHR was compromised against *P. syringae* pv *phaseolicola* NPS3121. In addition, growth of *P. syringae* p.v. *tabaci* and *P. fluorescens* for which Arabidopsis is a non-host, was supported on *nho1* plants (Lu *et al.,* 2001). Also, it was demonstrated that *NHO1* is required for resistance to the necrotrophic pathogen *B. cinerea* (Kang *et al.,* 2003): an indication of the generic role of *NHO1* in disease resistance. However, *NHO1* is only required for resistance against certain groups of pathogens, as the *nho1* does not support disease susceptibility to virulent *Pseudomonas* bacteria (Kang *et al.,* 2003).

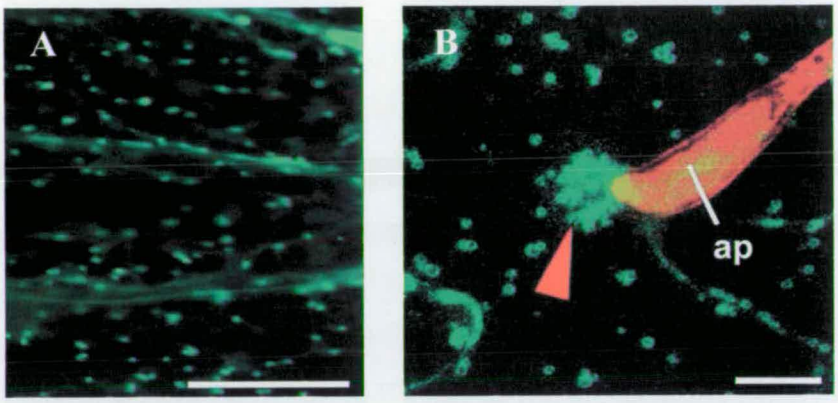


Figure 1.1. A) Confocal laser scanning microscopy of leaf epidermal cells in transgenic *pen2*-1 mutants expressing a functional PEN2-GFP fusion reveals association with vesicle-like bodies. Scale bar, 10 µm; B) Peroxisomes containing PEN2-GFP accumulate at *Bgh* entry sites (marked by red arrowhead). Fungal structures are stained in red by propidium iodide. Scale bar, 10 µm; ap, appressorium. Source: (Lipka *et al.,* 2005).

Besides, there are general elicitors which are released during initial interaction of both host and non-host pathogens. It has been shown these elicitors trigger receptor mediated defence in NHR interactions (Jones and Takemoto, 2004). In this context, activation of immunity system are likely to be in a non-specific fashion, equivalent to the recognition of PAMPs in the innate immune response of animals (Akira and Takeda, 2004). Examples of such PAMP-like elicitors in bacteria are flagella (Zipfel *et al.,* 2004), EF-Tu (Zipfel *et al.,* 2006) and lipopolysaccharide (Zeidler *et al.,* 2004b; Zeidler *et al.,* 2004a) and for fungi are β-glucan and chitin (Nurnberger *et al.,* 2004). Interaction between flg22 and FLS2 leads to accumulation of Reactive oxygen specific (ROS) and activation of mitogen-activated protein kinase (MAPK) cascade, in addition to induction of defence pathogenesis-related (*PR*) genes (Felix *et al.,* 1999; Nuhse *et al.,* 2000; Asai *et al.,* 2002). In this context, the role of non-functional effectors might not

be underestimated. In fact, a pathogenic bacterium for example, delivers more than 20 effectors through a type III secretion system (Buttner and Bonas, 2006) and only some of these proteins may be able to effectively manipulate plant cell function or suppress PAMP-triggered immune responses (Hauck *et al.*, 2003). Nevertheless, detection of non-functional effectors could activate plant immune system (Ellis, 2006).

R Gene Mediated Resistance

Subsequent to pathogen penetration, certain varieties of plant species express resistance to only particular races of pathogens, a phenomenon known as race-specific resistance (Parlevliet, 2002). This type of incompatible interaction follows gene-for-gene relationship (Flor, 1971). In the gene-for-gene model a single dominant gene (*R*) in the host encodes a protein that recognizes the protein of a matching dominant avirulence (*Avr*) gene expressed by the pathogen. This kind of resistance is usually marked by a hypersensitivity reaction (HR), which involves a wide range of active cell mechanisms including programmed cell death (PCD) at the site of penetration or infection (Parlevliet, 2002). On the other hand, mutation of either the plant R protein or pathogen AVR protein leads to the plant's failure to sense the pathogen and consequently results in susceptibility (Fig. 1.2). The stringent response and expression of the immune phenotype is the most attractive feature of *R* gene mediated resistance. However, the evolutionary potential of pathogens poses a great risk to overcoming the effect of *R* genes (McDonald and Linde, 2002).

In the gene-for-gene model a direct interaction was predicted between plant receptor and pathogen elicitor as it hypothesis especificity, however, this has attracted some questions. For example, there are only a few pieces of supporting evidence available for direct interaction.

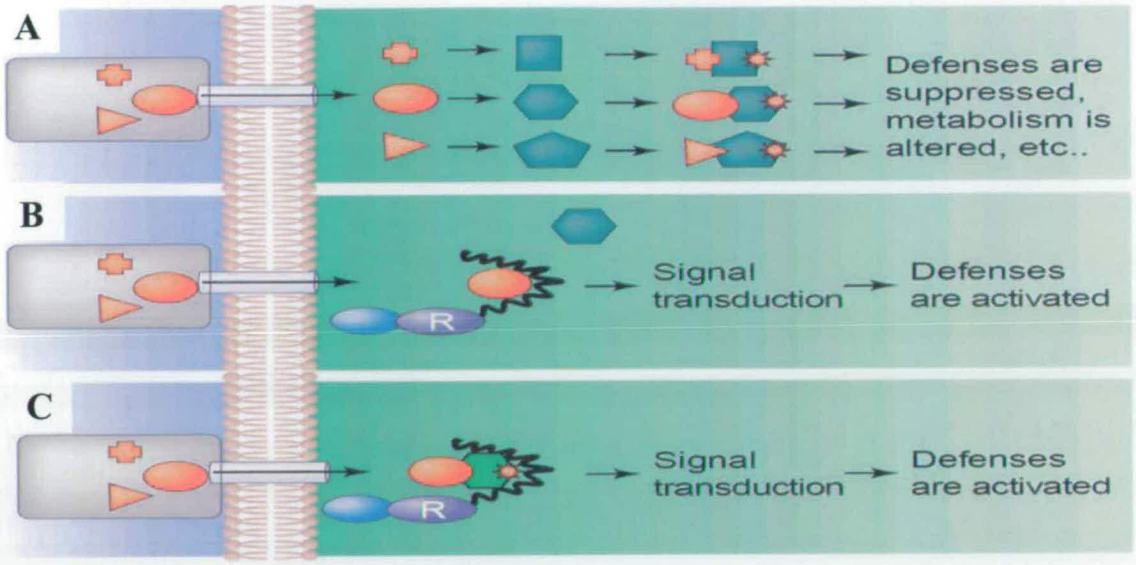


Figure 1.2. Interactions between pathogen Avr proteins and plant R proteins. A hypothetical pathogen (grey) has attached to a plant cell and is expressing a suite of virulence proteins (red). These proteins are translocated into plant cells via Type III secretion (bacteria) or other unknown mechanisms (fungi and oomycetes). Once inside, they target host proteins (green) that control defence responses, metabolism or other plant process that affect pathogen virulence (note that virulence proteins could also be targeted towards extracellular proteins); A) In this panel, the plant cell does not express an R protein that is capable of recognizing any virulence protein. Thus, the plant cannot detect the pathogen efficiently and defences are, at best, only weakly induced. Disease then results from the collective action of the virulence proteins; B) This panel depicts the classic receptor–elicitor hypothesis, in which an R protein directly binds a virulence protein. This recognition event activates a complex signal transduction network, which in turn triggers defence responses; C) This panel depicts the guard hypothesis, in which an R protein (guard) detects a modified host protein (guardee, red star), perhaps as a complex with the 'attacking' virulence protein. Source:(McDowell and Woffenden, 2003).

One example is the physical association that was demonstrated between the *Pto* gene in tomato conveys resistance to strains of *Pst* carrying the avirulence gene *avrPto* (Tang *et al.,* 1996). The other issue was raised regarding the limited number of available *R* genes in plants. As the direct R-Avr protein interactions fails to reason, how for example ~200 *R* genes in Arabidopsis are able to interact with copious numbers of pathogens and their diversity within their species, genus

and isolates. These insufficiencies of the direct interaction in gene-for-gene hypothesis were explained by an alternative theory named "guard hypothesis". The guard hypothesis suggests that many plant R proteins are activated indirectly by pathogen-encoded effectors through monitoring a limited number of pathogen effectors that play important roles in pathogenicity (Dangl and Jones, 2001). In this case, R protein interacts with another plant protein (the guardee) to activate resistance (van der Biezen and Jones, 1998). Thus, the guardee is targeted by the Avr protein and resistance is activated when the R protein detects Avr binding or modification of the targeted protein. An example studied in detailed, is the *RPM1-interacting protein4 (RIN4)* gene, a negative regulator of basal resistance (Mackey *et al.,* 2002). RIN4 protein interacts with at least three unrelated *P. syringae* type III effectors, AvrRpm1, AvrRpt2 and AvrB (Mackey *et al.,* 2003). The resistant protein RPM1 detects AvrB and AvrRpm1, through phosphorylation of RIN4 (Mackey *et al.,* 2002). In addition another plant protein, RPS2 which detects AvrRpt2 protein, physically interact with RIN4 at the plasma membrane in Arabidopsis. Delivery of AvrRpt2 into the host first induces disappearance of RIN4 then activates RPS2. The RIN4-AvrRpt2 interaction is independent of RPS2 (Mackey *et al.,* 2003).

Structure of R Proteins

The vast majority of Arabidopsis *R* genes encode proteins that contain a nucleotide-binding site (NBS) in their amino-terminal (N-terminal) and leucine-rich repeats (LRRs) in their carboxy-terminal (C-terminal) (Fig 1.3). The protein products of other *R* genes contain the conserved motif of NBS but lack LRRs (Meyers *et al.,* 2002). NBS-LRR proteins also play an important role in animal immune systems as they are related to the CARD-Nod (caspase recruitment domain nucleotide binding oligomerization domain) family (Inohara and Nunez, 2003). Mammalian NOD proteins are involved in recognition of microorganisms and act as cytosolic sensors to induce apoptosis (Inohara and Nunez, 2003).

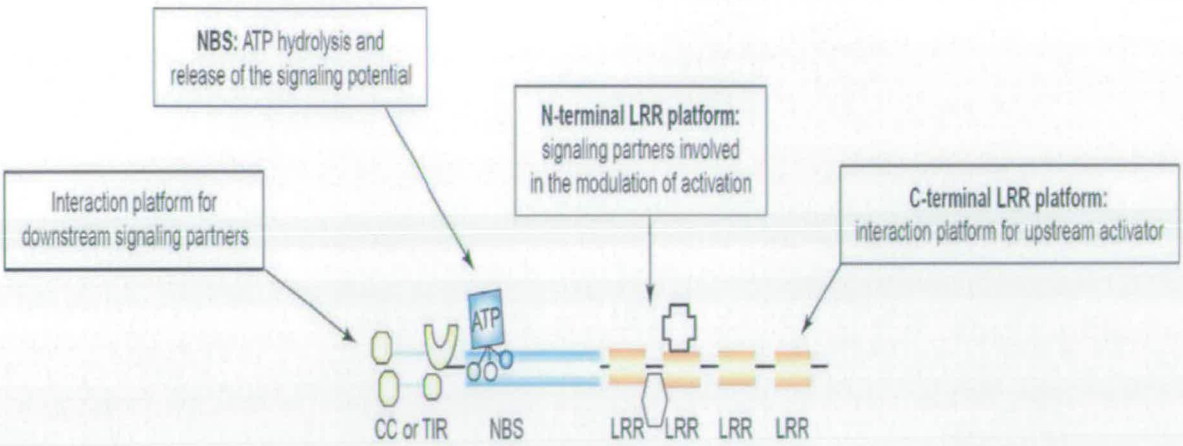


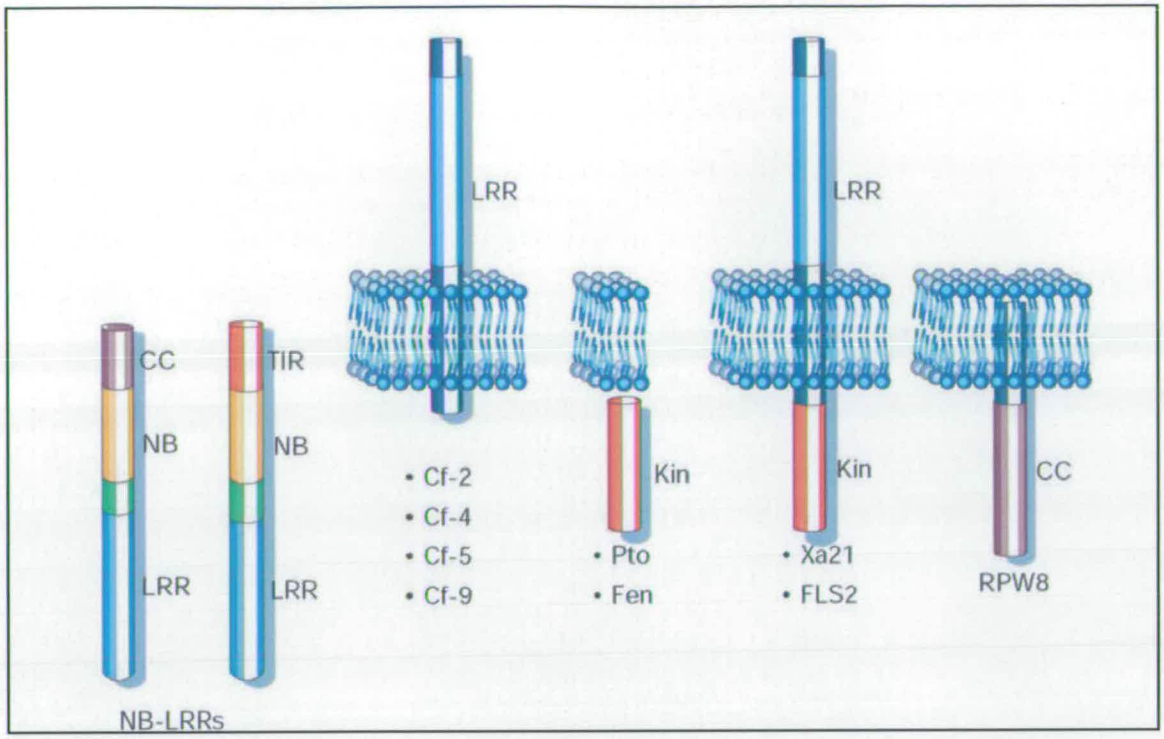
Figure 1.3. Domain structure of NBS–LRR proteins. A schematic representation of NBS–LRR proteins shows a domain-based platform for the assembly of various putative regulatory factors (i.e. CC, TIR, NBS and amino-terminal [N-terminal] LRR regions) necessary for controlled signalling. These domains also link to a possible intramolecular regulatory region on the carboxy-terminal (C-terminal) LRR. The cartoons in yellow represent putative interactors assembled on and carboxyl to the CC/TIR domains. The blue square represents ATP, but could also be GTP. The gray cartoons that are associated with the amino-terminal part of the LRR domain represent another set of putative interactors that might be positive regulators. Source: (Belkhadir *et al.,* 2004).

The diverse features of LRR domains and their presence in various proteins have led to the idea that they function as interaction sites for protein-protein binding with the ability to recognise specific pathogen effectors (Dangl and Jones, 2001; Deyoung and Innes, 2006). In addition, there are also suggestions that LRR domains act as a regulatory domain for disease resistance (Deyoung and Innes, 2006). Recombination of the *L2* allele (the flax rust resistance gene) in flax with the *L6* and *L10* alleles that encoded LRR of *L2* showed pathogen specificity of *L2* allele (Ellis *et al.,* 1999). Furthermore, interaction of the LRR-like domain of Pi-ta (resistance protein to rice blast pathogen) with AvrPita (Jia *et al.,* 2000) is another example that supports the specificity of protein-protein interactions

mediated by the LRR domain. Conversely, full length of RPS1-R (a protein conferring resistance to bacterial wilt) is required to interact with PopP2 (a type III effector) not just the LRR domain alone (Deslandes *et al.,* 2003), indicating that multiple domains are required for the detection of a pathogen effector protein. The regulatory role of the LRR domain was demonstrated by using a deletion assay for RPS2 and RPS5 proteins. Removal of the LRR domain caused constitutive activation of defence responses, while both of these plant proteins in full length are negative regulators of resistance (Deyoung and Innes, 2006). On the other hand, deletion of the LRR domain in the I-2 protein did not result in constitutive resistance activity (Tameling *et al.,* 2006). I-2 is the tomato NBS-LRR protein which confers resistance to the fungal pathogen *Fusarium oxysporum* (Ori *et al.,* 1997). The corresponding AVR protein for I-2 remains to be identified (Tameling *et al.,* 2006).

The nucleotide-binding-site (NBS) domain, which is a conserved motif in both animals and plants (Takken *et al.,* 2006), seems to be responsible for ATP binding. Activation of signal transduction begins by binding of ATP to the NBS, and this creates binding sites for downstream signalling molecules (Tameling *et al.,* 2006).

There are around 200 *R* genes in Arabidopsis that encode at least five different classes of Resistance proteins (Fig 1.4) (Dangl and Jones, 2001; Meyers *et al.,* 2002). NBS-LRR proteins are divided into two major classes based on the presence of domains in their N-terminus (Meyers *et al.,* 2003). The TIR domain has homology to the Toll and interleukin-1 receptor (Meyers *et al.,* 1999). Most NBS-LRRs proteins lacking the TIR domain have a α-helical coiled coil (CC) motif in their N-terminus (Pan *et al.,* 2000). The exact functions of these domains are not clear. However, it was suggested that TIR or CC domains may function in pathogen detection, regulation of NBS-LRR protein and the successive downstream defence pathways.



Figuer 1.4. Location and structure of the five main classes of plant disease resistance proteins. Xa21 and Cf-X proteins carry transmembrane domains and extracellular LRRs. The recently cloned RPW8 gene product carries a putative signal anchor at the N terminus. The *Pto* gene encodes a cytoplasmic Ser/Thr kinase, but may be membrane associated through its N-terminalmyristoylation site. The largest class of R proteins, the NB-LRR class, are presumably cytoplasmic (although they could be membrane associated) and carry distinct N-terminal domains. Source: (Dangl and Jones, 2001).

For instance, polymorphisms in the TIR domain of L (a TIR-NBS-LRR flax rust resistance protein) protein changed the specificity of *L* alleles in the interaction of *Linum usitatissimum* and *Melampsora lini* (flax- flax rust) (Luck *et al.,* 2000). The downstream signalling pathway of the TIR class of R proteins operates through ENHACED DESEASE SUSCEPTIBILITY (EDS)1 and PHYTOALEXIN DEFICIENT (PAD)4 , whereas CC-NBS-LRR proteins require NON-RACE SPECIFIC DISEASE RESISTANCE (NDR)1 for signal transduction (Aarts *et al.,* 1998). *EDS1* and *PAD4* encode lipase-like proteins

(Falk *et al.,* 1999; Jirage *et al.,* 1999), while *NDR1* encodes a glycosyl-phosphatidylinositol (GPI) anchored protein (Holt *et al.,* 2003).

The other four classes of *R* genes are less frequent and have different structures and localization (Fig 1.4). However, their products activate a similar range of defence mechanisms (Dangl and Jones, 2001). One of the well documented classes of *R* genes that includes tomato *Pto* gene, encode an intracellular Serine/Threonine kinase protein (Martin *et al.,* 1993). The *Pto* gene in tomato confers resistance to *Pst*, the causative agent of bacterial speck disease. Pto is an intracellular protein kinase which suggests a function in phosphorylation of other proteins involved in defence signalling (Martin *et al.,* 1993). *Pto*-like genes are highly conserved in many plant species and their encoded proteins are similar to the proteins of the *IRAK-1* (*IL-1 receptor-associated kinase*) in humans and the *Pelle* in *Drosophila* (Martin *et al.,* 1993; Pedley and Martin, 2003).

The third class of *R* genes were identified in tomato, which confer resistance to *Cladosporium fulvum*. The *Cf* genes encode membrane-anchored proteins largely composed of extracellular LRR domain (Jones *et al.,* 1994). The fourth class contains *Xa21* and *FLS2* genes. The *FLS2* gene requires flg22, a peptide representing the elicitor-active epitope of flagellin (Zipfel *et al.,* 2004). *Xa21* provides resistance to rice against race 6 of *Xanthomonas oryzae* pv *oryzae*. *Xa21* encodes a transmembrane receptor with a large extracellular LRR domain and an intracellular protein kinase domain (Song *et al.,* 1995).

In the last class of *R* genes, is the *RESISTANCE TO POWDERY MILDEW8* (*RPW8*) gene of Arabidopsis, cloned from accession Moscow-0 (Xiao *et al.,* 2001). This gene confers broad spectrum resistance to powdery mildew pathogens, *Erysiphe cruciferarum, E. cichoracearum* and *Odiuim lycopersici* (Xiao *et al.,* 2003). This resistance gene has no homology to any other previously described class of *R* genes and encodes a small putative transmembrane coiled-coil protein which lacks a nucleotide binding site and leucine-rich repeats (Xiao *et al.,* 2001).

Plant Basal Resistance

Fundamentally, a plant immune system is assembled on a defence structure composed of individual cells and systematic signals originating from an infected cell. This comprises PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI contributes to the immunity of plants at a very early stage which induces a weak but very important immune response (Jones and Dangl, 2006; Mackey and McFall, 2006). ETI is the second layer of the plant immune system that functions inside the cell by encoding a variable NB-LRR protein products to recognize pathogen effectors (Dangl and Jones, 2001; Jones and Dangl, 2006). Hence, ETI could be a synonym of gene-for-gene resistance (Jones and Dangl, 2006). Further to PTI and ETI, there is another part of plant immune system which remains active, even in susceptible plants, to restrict pathogen growth, and which is called basal resistance (Johnson, 1984). This part of resistance is also so-called non-race-specific, as the level of resistance in plant is similar to all races of the pathogen (Johnson, 1992). Basal resistance is not well defined, but is sometimes referred to as polygenic or horizontal resistance depending on different definitions or point of view (Hammond-Kosack and Parker, 2003; Johnson, 1984; Parlevliet, 2002). Either we call basal resistance, polygenic or horizontal resistance, this type of resistance does not break eventually unlike ETI, however levels of resistance may gradually decrease in basal resistance (Hammond-Kosack and Jones, 1997). Basal resistance is induced by general elicitors, whereas ETI relies on specific recognition (Hammond-Kosack and Parker, 2003). The presence of components in basal resistance which are able to recognise pathogen in a non-specific manner can be confusing, as the type of resistance triggered through PAMP elements such as flagellin or chitin often referred to basal resistance (Nurnberger *et al.,* 2004; Oh and Collmer, 2005). PAMP elements may or may not activate basal resistance. In an alternative and comprehensive concept, basal resistance may be triggered through microbe associated-molecular patterns (MAMP) (Mackey and

McFall, 2006; He *et al.,* 2006). MAMPs are any molecular sequence or structure in any pathogen-derived molecule which is sensed via direct interaction with plant receptors (Mackey and McFall, 2006). Plants respond to MAMPs irrespective of the pathogenicity of the pathogen (Ausubel, 2005). MAMP-mediated signalling is upstream of MAPKKK at the plasma membrane linked to the receptors and pathogen effectors such as AvrPto in transgenic Arabidopsis which blocks early MAMP signalling and enables non-host *P. syringae* to grow (Ausubel, 2005).

There are some other components of basal resistance that are not involved in early sensing of non-self molecules but playing important roles in orchestrating basal resistance. Mutations in *EDS1* (Wiermer *et al.,* 2005), *PAD4* (Xing and Chen, 2006), *Senescenc-Associated Gene* (*SAG*) *101* (Feys *et al.,* 2005), *NHO1* (Lu *et al.,* 2001) and *AtGSNOR1* (Feechan *et al.,* 2005) are some of the examples that compromised basal resistance as well as *R* gene-mediated resistance. These suggest some parts of the resistance signalling are conserved and shared between basal resistance and *R* gene mediated resistance.

Due to the location of the R protein, *R* gene mediated resistance is generally effective against biotrophic or hemi-biotrophic pathogens (Glazebrook, 2005). Thus, MAMP seems to take major contribution in basal resistance against necrotrophs.

Defence Signal Transduction

Pathogen perception triggers the plant defence system via a complex signalling network. Pathogens rapidly elicit a series of cellular responses such as the generation of reactive oxygen intermediates (ROIs) and nitric oxide (NO) (Delledonne *et al.,* 1998; Grant and Loake, 2000), expression of a particular array of defence-related genes and the accumulation of phytoalexins and defence related secondary metabolites (Zook *et al.,* 1998; Glazebrook and Ausubel, 1994;

Glazebrook, 2007; Glazebrook, 2001). Signalling molecules like salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play an essential role in triggering specific down stream signalling cascades.

1. Oxidative Burst

A rapid generation of reactive oxygen intermediates (ROI) at the site of infection, proceeding pathogen recognition is known as the oxidative burst (Doke *et al.,* 1996). ROIs, usually in form of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), play an important role in inhibiting the pathogen either by strengthening cell walls through oxidative cross-linking, direct killing the pathogen (Levine *et al.,* 1994) or through localized cell death (Loake, 2001). These radical ions are highly reactive and toxic with the capability of harming even the plant cells themselves by causing oxidative destruction. Sources of ROIs are plant organelles with highly oxidizing activity such as chloroplasts and mitochondria (Lamb and Dixon, 1997). ROIs can be generated by many different enzymes such as the NADPH oxidase complex at the plasma membrane (Grant and Loake, 2000) and are removed by a network of ROI-scavenging enzymes (Mittler *et al.,* 2004). A plasma membrane NADPH-dependant oxidase is the source of superoxide with similarity to the mammalian calcium-regulated NADPH oxidase (Torres *et al.,* 1998).

The extracellular superoxide dismutase (SOD) enzyme produces hydrogen peroxide (H_2O_2) by dismutation of O_2^- (Kliebenstein *et al.,* 1998), although H_2O_2 can also be produced from superoxide in a nonenzymatic fashion (Grant and Loake, 2000). Major sources of pathogen-induced ROIs in Arabidopsis are the respiratory burst oxidase homologues (*rboh* genes) related to the mammalian $gp91^{phox}$. Mutations in, *atrbohD* and *atrbohF* greatly reduced accumulation of H_2O_2 in response to the bacterial pathogen *Pst*DC3000(*avrRpm1*) and the oomycete pathogen *H. parasitica* isolate Emco5. This suggests a requirement for NADPH oxidases to generate hydrogen peroxide in defence response (Torres *et*

al., 2002). Hydrogen peroxide has the capability of passing through the cell membrane and being dispersed throughout the cell (Henzler and Steudle, 2000). H_2O_2 has a direct toxicity to both pathogen and plant cells. It acts in conjunction with nitric oxide to promote cell death and preventing further spread of the pathogen within the plant (Delledonne *et al.,* 2002; Morel and Dangl, 1997).

The presence of calcium binding domains containing EF hand motifs in Arabidopsis $gp91^{phox}$ suggests a possibility that elevation of cytosolic $[Ca^{2+}]$ promotes the oxidative burst through the activation of the NADPH oxidase complex in signal transduction (Keller *et al.,* 1998). Involvement of a Ca^{2+}-binding protein like calmodulin has been shown downstream of associated signals following ROI sensing (Bowler and Fluhr, 2000).

It has also shown that cell wall peroxidises generate hydrogen peroxide during an oxidative burst through a superoxide–binding intermediate that requires alkaline conditions (Bolwell, 1996). Attempted pathogen infection is followed by apoplastic alkalisation therefore rapid alkalinisation of the apoplast through plasma membrane ion channels may activate cell wall peroxidases (Bolwell *et al.,* 1999). Ascorbate and NADPH are two potential reductants of cell wall peroxidases (Bolwell *et al.,* 1999). Recent data suggest peroxidases play an important role in production of hydrogen peroxide during resistance responses to *Pst*DC3000 and *P. syringae* pv. *maculicola* strain ES4326 (Bindschedler *et al.,* 2006).

2. Nitric Oxide

The impact of NO on the activation of defence mechanisms has been shown in a wide spectrum of biological systems (Delledonne, 2005; Wendehenne *et al.,* 2004). This small and highly toxic gas acts as a ubiquitous component of signal transduction pathways and is also involved in several diverse physiological processes in plants (Delledonne *et al.,* 1998; Wendehenne *et al.,* 2004; Wang *et*

al., 2006). In animals the role of NO as a signalling molecule was addressed over a decade ago and was found to be generated by three distinct nitric oxide synthases (NOSs) (Mayer and Hemmens, 1997). In plants two NOS-like enzymes were identified. iNOS which is a pathogen-inducible enzyme (Chandok *et al.,* 2003) and AtNOS, a hormone-activated NOS (Guo *et al.,* 2003) both from Arabidopsis. The nitrate reductase (NR) enzyme which is located in the cytosol plays a key role in nitrogen assimilation in plants and produces NO by catalysing the reduction of nitrate (Yamasaki *et al.,* 1999). The sequences of the two recently identified Arabidopsis NOS genes are not similar to known mammalian *NOS* genes, however, both plant and animal *iNOS* genes are induced in response to pathogens (Zeidler *et al.,* 2004a). In addition the plant *iNOS* requires both Ca^{2+} and calmodulin for activity whereas the animal iNOS activity is independent of intercellular free Ca^{2+} (Guo *et al.,* 2003).

It was also demonstrated that plants are able to synthesis NO through nonenzymatic reactions, such as formation of NO_2 by carotenoids in the presence of light (Wendehenne *et al.,* 2001; Wojtaszek, 2000). NO regulates activation of different proteins, through *S*-nitrosylation (Wang *et al.,* 2006). *S*-nitrosylated proteins are created when a cysteine thiol reacts with NO and form an *S*-nitrothiol (SNO) bond (Stamler *et al.,* 1992). Formation of SNO subsequently modulates protein function. A central role for *S*-nitrosylation has been demonstrated in plant immunity (Feechan *et al.,* 2005). In the absence of *AtGSNOR1* both accumulation of SA and SA signalling were reduced, suggesting that *S*-nitrosylation controls nodes both upstream and downstream of SA accumulation (Feechan *et al.,* 2005).

On the other hand, balanced accumulation of NO and ROI prompts development of the HR (Delledonne *et al.,* 1998; Delledonne *et al.,* 2001). The triggered HR via this synergy is dependent on the interaction of NO with the H_2O_2 which is generated from O_2^- by superoxide dismutase. NO does not interact directly with O_2^-. Thus, O_2^- is the primary ROI signal for pathogenesis induction of glutathione *S*-transferase, and the rates of production and dismutation of O_2^- during the oxidative burst is fundamental for HR induction (Delledonne *et al.,* 2001).

Nitric oxide is also required for PAMP-triggered stomatal closure. It was shown that PAMP perception triggers stomatal closure which generates a barrier against bacterial infection (Zipfel *et al.,* 2004). Stomatal closure in abiotic stress, in addition to other hormones and downstream signals, requires guard-cell-specific OST1 (OPEN STOMATA1), a Ca^{2+}-independent protein kinase (Mustilli *et al.,* 2002). Treatment of *ost1-2* mutants with bacterial PAMPs, flg22 and LPS did not induce stomatal closure. Whereas both PAMPs i.e. flg22 and LPS, reduced stomatal aperture in wild type following a rapid nitric oxide burst in guard cells. While Nω-nitro-L-arginine (L-NNA), a NOS inhibitor, effectively prevented flg22 and LPS to induce stomatal closure in wild-type plants (Melotto *et al.,* 2006).

3. Salicylic Acid Signalling

Perhaps the roles of salicylic acid (SA) have been best studied as a pivotal signalling molecule in the activation of the plant defence system (Durrant and Dong, 2004; Delaney *et al.,* 1994). SA is a phenolic hormone that increases in the cell, either locally in the infected leaves or systematically, subsequent to pathogen attack (Durrant and Dong, 2004). The increased levels of SA activate the transcription of defence genes and the development of an efficient pathogen resistance response, while its absence can cause susceptibility (Delaney *et al.,* 1994). For instance, the tobacco and Arabidopsis *NahG* transgenic plants show enhanced susceptibility to a broad range of fungal, bacterial and viral pathogens. These plants express the bacterial *NahG*, encoding a salicylate hydroxylase, which converts SA into catechol (Delaney *et al.,* 1994). Two Arabidopsis lines carrying *SA induction-deficient* (*sid*) *1* and *sid2* mutations convey enhanced susceptibility to the virulent pathogens *Pst*DC3000 and *H. parasitica*. Both of these mutants demonstrate the importance of SA in resistance. The *sid1* gene is allelic with *enhanced disease susceptibility* (*eds*) *5* mutation (Nawrath and Metraux, 1999). *SID2* is involved in the conversion of chorismate to

isochorismate and encodes a chloroplast-localized isochorismate synthase. Subsequently, isochorismate is assumed to be converted to SA by an isochorismate pyruvate lyase (Wildermuth *et al.,* 2001).

Rapid accumulation of SA at the site of pathogen attack induces a range of defence responses from a local reaction in the form of cell death to systemic immunity in response to a range of biotrophic pathogens termed systemic acquired resistance (SAR) (Klessig and Malamy, 1994). Salicylic acid is a necessary molecule for the stimulation of the SAR signals which spreads from the infected sites to systemic tissues through the phloem (Molders *et al.,* 1996). However, it was shown in tobacco and cucumber that SA is unlikely to be the primary mobile molecule for exportation from the infected leaf to other part of the plant (Molders *et al.,* 1996; Vernooij *et al.,* 1994). Inoculation of Arabidopsis plants with virulent *Pst* subsequent to pre-treatment with avirulent *Pst* strain caused rapid cell death (Cameron *et al.,* 1994). Likewise, application of exogenous SA on tobacco plants triggered rapid cell death when infected with *Erwinia carotovora* (Palva *et al.,* 1994). The cell death triggered through activated SAR is similar to HR, while both are regulated via the oxidative burst (Greenberg, 1997). The increased cell death in SAR is specific to bacterial and fungal pathogens since it was reduced when infected with avirulent viruses (Gaffney *et al.,* 1993). Application of either exogenous SA or its chemical analogue 2,6-dichloroisonicotinic acid (INA) or benzotiadazole (BTH) to plants activates SAR (Lawton *et al.,* 1996; Uknes *et al.,* 1992).

To uncover the components involved in the transduction of SA signalling, a screening carried out on Arabidopsis mutants to identify those genotypes that failed to activate the SA- or INA-responsive chimeric reporter gene expression. This research has led to the isolation of *Nonexpressor* of *PR* (*NPR*)1 or *Noninducible Immunity* (*NIM*) 1 (Cao *et al.,* 1994). This gene functions downstream of SA, as *npr1/nim1* mutations block the expression of *Pathogenesis Related* (*PR*) genes in response to SA (Cao *et al.,* 1994; Delaney *et al.,* 1994). Sequence analysis of *NPR1* and its mutant alleles have shown that NPR1 contains

a functionally important ankyrin repeat domain (Cao *et al.,* 1997). Ankyrin plays a role in mediating protein–protein interactions (Sedgwick and Smerdon, 1999). Multiple yeast two-hybrid screens also suggest that NPR1 might interact with TGA transcription factors to mediate *PR* gene expression (Zhang *et al.,* 1999; Despres *et al.,* 2000). Further to modulating *PR* genes expression, *NPR1* directly regulates the expression of the protein secretory pathway genes, which is essential for SAR (Wang *et al.,* 2005).

EDS1, PAD4 and *NDR1* genes function upstream salicylic acid signalling. Mutation in either of these genes compromise basal resistance and application of exogenous SA enhance their expression (McDowell *et al.,* 2000; Zhou *et al.,* 1998; Jirage *et al.,* 1999). In addition , EDS1 and PAD4 transduce ROS-derived signals in disease resistance signalling (Rusterucci *et al.,* 2001) which this was evidenced in *SAG101* mutant as a role beyond their activities on regulation of SA (Wiermer *et al.,* 2005). The Arabidopsis *SAG101* is involved in both *R* gene mediated (TIR-NBS-LRR) and basal resistance, which together with *PAD4* takes major contribution in basal defence mechanism. Arabidopsis genotypes contained both *sag101* and *pad4* mutant genes allowed more pathogen development compare to *eds1* when infected with non-host pathogen *Bgh* or *Erysiphe pisi* (*Ep*) (Wiermer *et al.,* 2005). Although an essential role for PAD4-EDS1 was demonstrated in induction of SA-dependent resistance, but they are also involved SA-independent responses (Bartsch *et al.,* 2006). Downstream PAD4-EDS1, the *MAP Kinase* (*MAPK*)*4* down regulates SA accumulation but stimulates JA and ET signalling pathway to *Alternaria brassicicola* (Wiermer *et al.,* 2005; Petersen *et al.,* 2000).

SA signalling leads to expression of specific *PR* genes, including *PR-1*, *PR-2, PR-3* and *PR-5* as marker genes for systemic immunity (Ryals 1996). *PR-2* encodes β-1, 3-gucanase and *PR-3* encodes chitinase while the functions of the other *PR* genes are poorly understood (Hammond-Kosack and Jones, 1997). Although, either pathogen infection or application of exogenous SA or its chemical analogue induced SAR, they induced different set of *PR* genes.

Treatment of tobacco plants with exogenous SA, INA or BTH induced *PR-1* whereas *PR-2* and *PR-3* were expressed upon pathogen infection (Vidal *et al.*, 1997).

4. JA Signalling

In response to pathogen infection, jasmonic acid (JA) as a signalling molecule is produced and accumulates both in local and systemic tissues (Penninckx et al. 1996). Jasmonic acid signalling is also involved in modulating different plant growth and developmental characteristics, such as pollen development, anther dehiscence, abscission and senescence as well as many abiotic stresses (Turner et al 2002, Loake and Nuremberg 2003). Jasmonic acid belongs to a family of fatty acids which is derived from oxygenated linolenic acid (LA) through octadecanoic pathway (Vick and Zimmermand 1984). Jasmonic acid also stimulates expression of plant defence genes encoding antifungal thionins (Bohlman and Apel, 1991).

Evidence for the role of JA in pathogen resistance predominantly came from analyses of Arabidopsis mutants affected in biosynthesis or responsiveness to JA. The JA-response mutant *coronatine insensitive* (*coi*)*1* has been documented as displaying enhanced susceptibility to the necrotrophic fungi *A. brassicicola* and *B. cinerea* (Thomma *et al.*, 1998; Thomma *et al.*, 1997), and the bacterial pathogen *E. carotovora* (Norman-Setterblad *et al.*, 2000). *COI1* encodes an F-box motif with an LRR domain which perceive pathogen coronatine (Xie *et al.*, 1998) and belongs to the ubiquitin-ligase complex SCF^{COI1}. This complex regulates expression of JA-responsive genes by making their repressors for ubiquitin-mediated destruction (Devoto *et al.*, 2002).

Another JA insensitive mutant of Arabidopsis, *jar1*, allows enhanced levels of growth of virulent *Pst*DC3000 (Pieterse *et al.*, 1998). *JAR1* encodes an adenylating enzyme that controls JA by modifying its activity (Staswick *et al.*, 2002). Furthermore, both *jar1* and the fatty acid desaturase (*fad3*, *fad7*, *fad8*)

triple mutants, which are defective in JA biosynthesis, exhibit susceptibility to normally non-host soil-borne *Pythium* sp. (Vijayan *et al.,* 1998). This data indicates that JA could be involved in non-host resistance against this class of pathogens. Jasmonic acid also contributes to basal resistance against insects (McConn *et al.,* 1997). Despite these findings and the isolation of both *COI1* and *JAR1* neither seems to be a receptor for JA evidently. On the other hand, *JAR1* is not required for all JA responses cause unlike *coi1* plants, *jar1* mutants are fertile (Devoto and Turner, 2003).

In the plastid, a lipolytic enzyme, DEFECTIVE IN ANTHER DEHISCENCE (DAD)1, releases linolenic acid (LA), which later on is oxygenated and turns to lipoxygenase (LOX) (Ishiguro *et al.,* 2001). Then, ALLEN OXYDE SYNTHASE (AOS) and ALLEN OXYDE CYCLASE (AOC) convert LOX to 12-oxo-phytodienoic acid (OPDA) which is catalyzed to jasmonic acid or MeJA through β-oxidation steps. Jasmonic acid contains a carboxyl group (R-COOH) on its root, which can be supplemented with a methyl group (CH_3) and convert JA to MeJA (R-$COOCH_3$) (Devoto and Turner, 2003; Ishiguro *et al.,* 2001). In Arabidopsis, JA and MeJA regulate expression of the genes encoding cell wall proteins and phytoalexin biosynthesis (Gundlach *et al.,* 1992). Moreover, application of exogenous MeJA promotes resistance to a number of necrotrophic pathogens in Arabidopsis (Thomma *et al.,* 2000).

The genes encoding antifungal proteins such as *PLANT DEFENSIN* (*PDF*)*1.2*, *THIONIN* (*THI*)*2.1* and *CHITINASE* (*CHI*)*B* are also induced by JA (Reymond and Farmer, 1998). AOS is a crytochrome P450 enzyme of the CYP74A family (Laudert *et al.,* 1996) which plays a major role in regulation of octadecaniod signalling (Laudert and Weiler, 1998). In Arabidopsis, *AOS* is rapidly induced upon wounding or insects attack and its level of expression steadily increases for up to 48 hours post stress (Laudert and Weiler, 1998). *OsAOS2* is induced in rice leaves after inoculation by rice blast, *M. grisea*. This was accompanied by enhanced activation of PR proteins, suggesting a role for JA in regulation of PR proteins and resistance to rice blast disease (Mei *et al.,* 2006). Moreover, a

contribution of JA-based signalling in systemic immunity was recently reported. JA rapidly accumulates in phloem exudates of the challenged leaves with avirulent strains of *Pst* preceding increase in SA. Suggesting a possible role for JA to initiate resistance signal at infection site for activation of systemic defence (Truman *et al.,* 2007).

5. Ethylene Signalling

Ethylene (ET) is able to influence many aspects of plant growth and development such as germination, growth and differentiation, flower and leaf senescence, leaf abscission, fruit ripening, root nodulation, programmed cell death, and responsiveness to stress and pathogen attack (Johnson and Ecker, 1998; Ecker and Davis, 1987; Thomma *et al.,* 1999). Accumulation of ET is immediately initiated upon wounding or exogenous application of JA. It has also been demonstrated that similar types of defence genes were induced by JA and ET signalling pathways (Lorenzo and Solano, 2005; Dong, 1998). However, JA and ET can also have antagonistic interactions in the activation of defence gene expression (Xu *et al.,* 1994). Mutations in genes are required for ET signalling cause impaired defence responses and a subsequent increased susceptibility to some necrotrophic pathogens (Knoester *et al.,* 1998; Berrocal-Lobo and Molina, 2004; Berrocal-Lobo *et al.,* 2002).

Several mutants have been identified with defects in ET signalling including *ethylene receptor* (*etr*)*1* , *etr2, ethylene insensitive* (*ein*)*2, ein3, ein4, ein5,* and *ein6* (Guo and Ecker, 2004). Moreover, a number of *ET response factor* (*ERF*) genes are though to be functionally involved in disease resistance by promoting expression of defensin genes (Berrocal-Lobo *et al.,* 2002). Inoculation with necrotrophic pathogens induced *ERF1* expression. Moreover, constitutive expression of *ERF1* in transgenic plants enhanced resistance to several necrotrophic fungi (Berrocal-Lobo *et al.,* 2002; Berrocal-Lobo and Molina, 2004). Exogenous application of either ET or JA induces expression of *ERF1*.

Conversely, any mutations that impede either of these signalling pathways i.e. *ein2* or *coi1* prevents the induction of *ERF1* expression and its targets (Berrocal-Lobo and Molina, 2004). These results suggest a core signalling role for *ERF1* in integrating JA and ET pathways in plant defence, which acts downstream of the intersection between these two pathways (Lorenzo *et al.,* 2003).

EIN2 is a membrane-associated protein with sequence similarities to the NRAMP family of metal transporters (Alonso *et al.,* 1999). The *ein2* mutant displays an increased susceptibility to *E. carotovora* and decreased disease symptoms to *Xanthomonas campesteris* (Dong, 1998) suggesting that *EIN2* is an essential component in ET signalling during plant-pathogen responses.

6. Cross Talk between Defence Signalling Pathways

The effectiveness of the plant defence system is largely dependent on the cross-talk between signal transduction pathways. The specific pathogen or given elicitor determines the potential synergism or antagonism between defence signalling pathways (Fig.1.5) (van Loon *et al.,* 2006; Bostock, 2005). While SA-dependent pathways mainly mediate resistance to biotrophic pathogens, the JA and ET pathways provide resistance to necrotrophics (Glazebrook, 2005). However, there is growing number of studies providing evidence for both synergistic and antagonistic interaction between these pathways (Glazebrook, 2001; Dodds and Schwechheimer, 2002; Kunkel and Brooks, 2002). Microarray experiments showed a synergistic interaction between JA and SA where an overlapping set of genes were upregulated by application of either SA or JA (Li *et al.,* 2004; Schenk *et al.,* 2000). In contrast, there are also a series of studies demonstrating antagonistic interaction between JA and SA (Schenk *et al.,* 2000; Glazebrook *et al.,* 2003; Spoel *et al.,* 2003). For example, exogenous application of SA suppresses JA-mediated gene expression (Gupta *et al.,* 2000). It seems that cross-talk between defence pathways fine tunes plant signal transduction leading to activation of an optimal defence response.

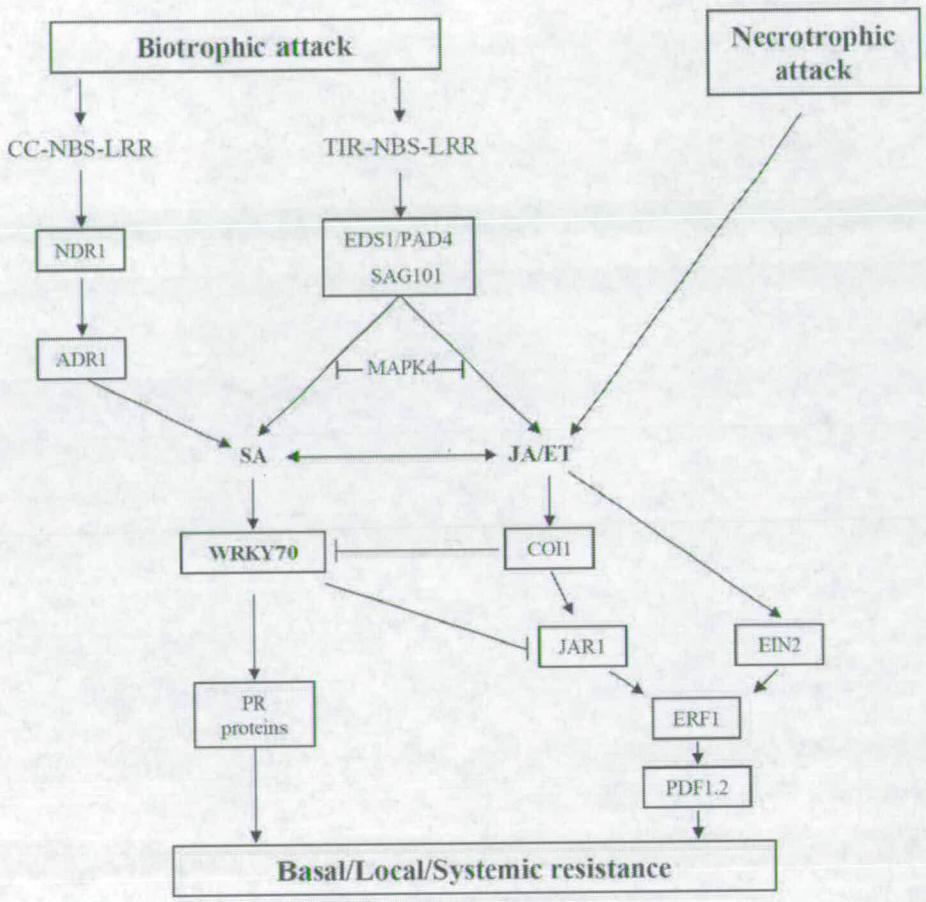


Figure 1.5. Defence signalling pathways. A simplified model for defence signals mediated against pathogens with different life styles and their cross linking. Biotrophic pathogens are mainly inhibited by SA dependent responses, whereas necrotrophs activate JA and ET defence pathways.

Recently a role was identified for the transcription factor WRKY 70 in modulating the selection of the signalling pathways (Eulgem, 2006; Li *et al.*, 2006). WRKY proteins are transcriptional regulators comprising a large gene family, a conserved domain WRKY at the N-terminal together with a zinc-finger motif (Eulgem *et al.*, 2000; Eulgem *et al.*, 1999). WRKY70 upregulates SA-related genes and suppresses expression of JA-responsive genes. Exogenous

application of SA or JA, upregulated or downregulated expression of *WRKY70,* respectively (Li *et al.,* 2004). Overexpression of *WRKY70* compromised resistance to the necrotrophic pathogen *A. brassicola* (Li *et al.,* 2006). During defence signalling, NPR1 is functionally linked to WRKYs (Spoel *et al.,* 2003). It is notable that JA signalling is essential for resistance of Arabidopsis to this necrotroph pathogen (Thomma *et al.,* 2000).

On the other hand, overexpression of WRKY70 in *wrky70* mutants confers resistance to *E. carotovora* and *Pst*DC3000 (Li *et al.,* 2006). WRKY 70 functions downstream of NPR1 in the SA signalling pathway (Li *et al.,* 2004). MAPK4 is implicated in antagonism between SA and JA defence signalling as a positive or negative regulator of JA or SA signalling, respectively (Petersen *et al.,* 2000). Loss of *MPK4* function compromised resistance to *A. brassicola* (Brodersen *et al.,* 2006). On the other hand, double mutants of *mpk4/eds1* or *mpk4/pad4* greatly suppressed SA accumulation and *PR1* gene expression in response to *Pst*DC3000. Whereas SA accumulation and *PR1* expression strikingly increased in *mpk4* single mutant compare to wild-type and the double mutant plants (Brodersen *et al.,* 2006).

Wheat Diseases

Wheat is a key crop throughout the world, which has been cultivated for more than 6000 years. Along with its importance, wheat is vulnerable to diseases and its productivity can be seriously affected by several fungal diseases such as powdery mildew, rusts and leaf blotch (Oerke, 2006). In this section we will describe the leaf rust and the powdery mildew diseases of wheat. I have used these two pathogens extensively in our experiments as they are both very important diseases of wheat.

1. Leaf Rust

The wheat leaf rust pathogen (*Puccinia triticina* Eriks & Henn) belongs to the Pucciniaceae family, Uredinales order and Basidiomycete class (Anikster *et al.*, 1997). This pathogen is highly specialized with a narrow host range. It was first named *P. recondita* f.sp. *tritici*, however, the latest morphological studies showed that *P. recondita* is different from wheat leaf rust, thus *P. triticina* was the suggested name for the casual agent of leaf rust in wheat (Savile, 1971; Anikster *et al.*, 1997).

Small, orange pustules are the prominent feature of disease caused by leaf rust. Urediospores erupt through the epidermis and form a grainy orange dust on the leaf surface around the pustule. Sporulation primarily occurs on the upper leaf surface, although leaf sheaths can also be infected on extremely susceptible cultivars under favourable conditions (Fig. 1.6).

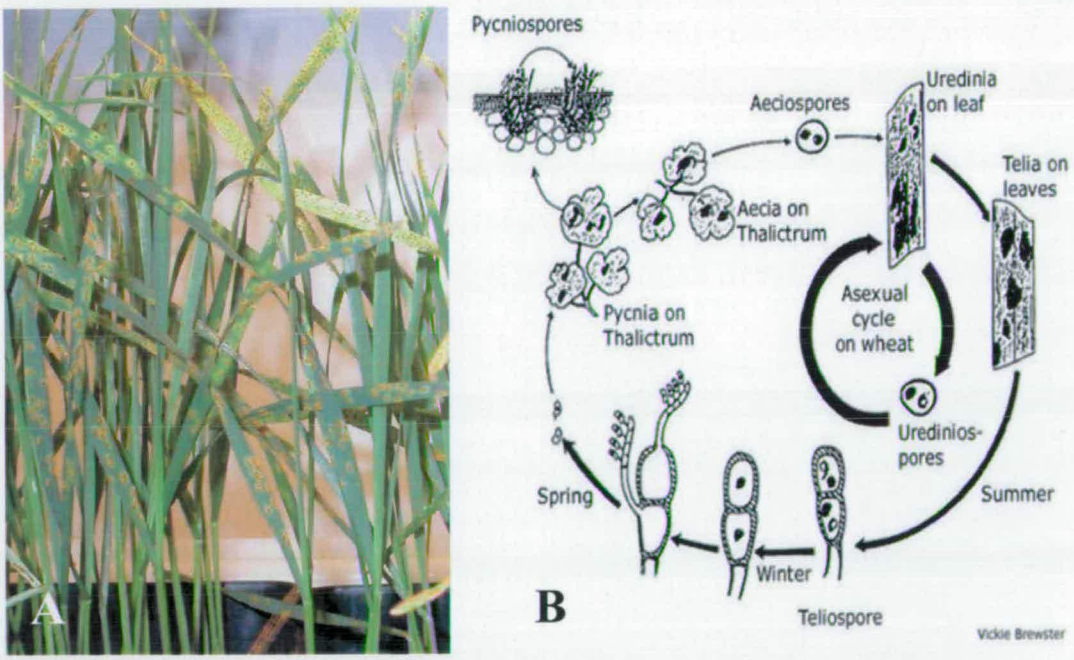


Figure 1.6. A) Leaf rust infection on the susceptible wheat cultivar-Vuka; B) Life cycle of *P. triticina*. (Image B: Art work of Vicie Brewster, Cereal Disease Laboratory, U of Minnesota; http://www.cdl.umn.edu/introduction/life_cyc.html).

In some cases, pustules are surrounded by a narrow yellow or white halo. During later stages, instead of orange spores pustules begin to produce dark black spores. Although leaf rust may initiate tiny orange spots on floret and heads, it does not form large, open pustules on these organs. This helps to distinguish leaf rust from stem rust (*P.graminis* f.sp. *tritici*) which is uncommon and usually is observed late in the season as it requires warmer temperatures. Unlike yellow rust (*P.striiformis*), leaf rust pustules distributed randomly across the leaf while yellow rust has narrow yellow stripes of pustules. Yellow rust requires relatively cool weather (Daamen *et al.,* 1992; Ellison and Murray, 1992). The disease develops rapidly and spreads typically uniformly across a field at temperatures between 10° and 30°C and to some extent wherever wheat is grown (Table 1.1). The main losses in yield are due to reduced floret set and grain shrivelling. In highly susceptible genotypes florets or even the whole plant can be killed by early epidemics (Sharma and Sharma, 2001). Leaf rust damage can be between 10 to 30% yield loss and in severe epidemics could be more.

Table 1.1. Environmental conditions required for wheat leaf rust

Stage	Temperature (°C)			Light	Free water
	Minimum	Optimum	Maximum		
Germination	2	20	30	Low	Essential
Germling	5	15-20	30	Low	Essential
Appressorium	-	15-20	-	None	Essential
Penetration	10	20	30	No effect	Essential
Growth	2	25	35	High	None
Sporulation	10	25	35	High	None

(Source: Saari and Prescott, 1985).

Most of the severe epidemics occur when uredinia survive the winter at the threshold level on the self-sown wheat near farms. The other possibility is that the spring wheat receives an exogenous inoculum during its early growing stages, usually before heading (Roelfs and Martell, 1984; Roelfs *et al.,* 1972). Infection of the flag leaf before anthesis causes the highest yield loss (Seck *et al.,* 1985).

P. triticina is able to survive on a relatively wide range of environmental conditions, which is very similar to the living condition of the wheat leaf (Table 1.2). Availability of free water is required up to penetration stage which can take place within three hours post infection at an optimum temperature of 20^{oC}, however, longer suitable conditions leads to more penetrations. Dew is the usual source of free water. Longer dew periods are needed at cooler temperatures. Rain favours pathogen infection by depositing spores from the air onto plants and also by increasing the humidity. On the other hand, rain can also wash landed spores from the plant surfaces (Geagea *et al.,* 2000).

Urediospores start to germinate around 30 minutes after being in contact with free water at temperatures of 15° to 25°C. The germ tube grows along the leaf surface to locate a stoma. Then an appressorium is formed and followed instantly by the development of a penetration peg and a sub-stomatal vesicle from which primary hyphae initiate. Then a haustorial mother cell develops against the mesophyll cell which is directly penetrated. The haustorium is formed inside the living host cell in a compatible host-pathogen interaction. Further haustorial mother cells and haustoria are formed subsequent to the development of secondary hyphae. In an incompatible host-pathogen response, haustoria fail to develop or develop at a slower rate (Martinez *et al.,* 2004; Niks and Dekens, 1991). Leaf rust is able to complete its life cycle on a susceptible cultivar between 7 and10 days at constantly optimum temperatures. The latent period is longer at lower temperatures (10° to 15°C).

Table 1.2. Importance of wheat rusts in different epidemiological zones

Zone	Leaf rust		Stem rust		Stripe rust	
	Current	**Historical**	**Current**	**Historical**	**Current**	**Historical**
Africa						
North	**Major**	**Major**	**Local**	**Major**	**Local**	**Local**
East	**Local**	**Local**	**Major**	**Major**	**Major**	**Major**
Southern	**Local**	**Local**	**Local**	**Major**	**Local**	**Rare**
Asia						
Far East	**Local**	**Local**	**Local**	**Major**	**Major**	**Major**
Central	**Major**	**Major**	**Minor**	**Minor**	**Local**	**Local**
South	**Local**	**Major**	**Minor**	**Major**	**Local**	**Local**
Southeast	**Major**	**Major**	**Minor**	**Minor**	**Rare**	**Rare**
West	**Local**	**Local**	**Local**	**Major**	**Major**	**Major**
Australia, New Zealand	**Local**	**Local**	**Local**	**Major**	**Local**	**Rare**
Europe						
East	**Major**	**Major**	**Minor**	**Major**	**Local**	**Local**
West	**Local**	**Major**	**Minor**	**Major**	**Major**	**Major**
North America	**Major**	**Major**	**Minor**	**Major**	**Local**	**Local**
South America	**Major**	**Major**	**Local**	**Major**	**Local**	**Local**

Major = severe losses without the cultivation of resistant varieties; Minor = usually occurs, but of little significance; Local = only occurs in a small part of the region, losses in these areas may be occasionally severe if susceptible cultivars are grown; Rare = not present, rarely seen, or as in Australia and New Zealand, recently introduced (Source: Saari and Prescott, 1985).

Maximum sporulation can be seen about four days following initial sporulation (at about 20°C) and lasts up to three weeks or as long as the wheat leaf remains alive (Niks and Dekens, 1991). The teliospores are formed under the epidermis and stay in the leaves while senescence happens and can be dispersed or moved by wind, animals or humans to considerable distances. Basidiospores do not play an important role for long distance distribution as they are light sensitive and only released under humid conditions. Long-distance transport has not been reported for aeciospores although they look similar to urediospores regarding their ability to be transported by wind. Urediospores are the main source of infection. They are produced in large amounts and can be blown by the wind for considerable distances. Urediospores can survive for several weeks away from host plants. They can also resist temperature below zero if the content of their moisture is less than 30 percent (Kolmer, 2005).

Potential hosts for wheat leaf rust could be wild species of the genera *Triticum* and *Aegilops* and the related species of the *Agropyron* complex and *Secale*. The primary host of *Puccinia triticina* (*Ptr*) is wheat (*Triticum aestivum* L.) however it is threatening for triticale (X *Triticosecale* Wittmack), the man-made crop invented by crossing wheat and rye (Skovmand *et al.,* 1984; Zillinsky *et al.,* 1980). *Ptr* is also able to attack *T. speciosissimum* including the durum wheat by producing its sexual gametes (pycniospores and receptive hyphae) (Anikster *et al.,* 1997). The later hosts are not important as a source of direct inoculums to wheat, but the story might point toward a mechanism for genetic exchanges between races and perhaps as a source of genetic variation in the pathogen populations.

2. Powdery mildew

Powdery mildew is one of the major limiting factors in wheat production around the world. The casual agent of this destructive foliar disease in wheat is an obligate biotrophic fungus, *Blumeria graminis* f.sp. *tritici* (*Bgt*), formerly named *Erysiphe graminis* f.sp. *tritici* (Lipps and Madden, 1988). Yield losses range between 5 and 45% in maritime or sub-continental climates (Audsley *et al.,* 2005; Gaunt, 1995).

Disease symptoms first appear as a white cotton-like spots on the upper surface of leaves, and as the disease ages the colour of pustules change from white to pale brown. Host infection involves a complex process, which is initiated immediately after conidia land on the leaf surface. To infect host, the conidia initiats a primary germ tube (PGT) and subsequently an appressorial germ tube (AGT) (Kunoh *et al.,* 1985; Takamatsu *et al.,* 1982). The PGT emerges 0.5-2 hours after inoculation and its function is to recognize an appropriate inductive surface. If PGT recognition fails then it will become non-functional and remain short (Carver *et al.,* 1999). In contrast to the rust fungi, colony development and generation of haustoria result from the ramifying hyphae growing on the leaf surface. Only haustoria develop within the invaginated plasma membrane of host epidermal cells (Fig 1.7).

When the PGT successfully makes contact with plant then an AGT emerges 3-3.5 hours later (Kunoh *et al.,* 1978). About 12-15 hours after inoculation, from beneath an AGT a penetration peg appears which attempts to penetrate the underlying epidermal cell to form a haustorium. The haustorium is normally elliptical with long finger shaped appendages protruding from both ends.

If the first penetration attempt fails by a germtube, a second germtube may initiate usually from the opposite site of the frist germtube. Although germination and formation of appressoria are light independent, the maturation of appressoria is light sensitive and could be inhibited by both darkness and high intensity of light. Following penetration the tip of the penetration peg enters the epidermal

cell and forms a haustorium (Fig. 1.7). This specialized absorption structure takes up nutrients from wheat to give rise to the formation of secondary hyphae and subsequently conidiophores on the surface. The pathogen life cycle is completed in 3-5 days on susceptible cultivars. Mild temperature, relatively high humidity (not free water) and heavy application of nitrogen fertilizer favours *Bgt* epidemics (Kunoh *et al.,* 1979; Carver *et al.,* 1999). Following infection, reduction in wheat photosynthetic area and vigour occurs due to the extensive utilization of nutrients and water by the pathogen, which can trigger plant death in sever situations (Alexopoulus, 1996).

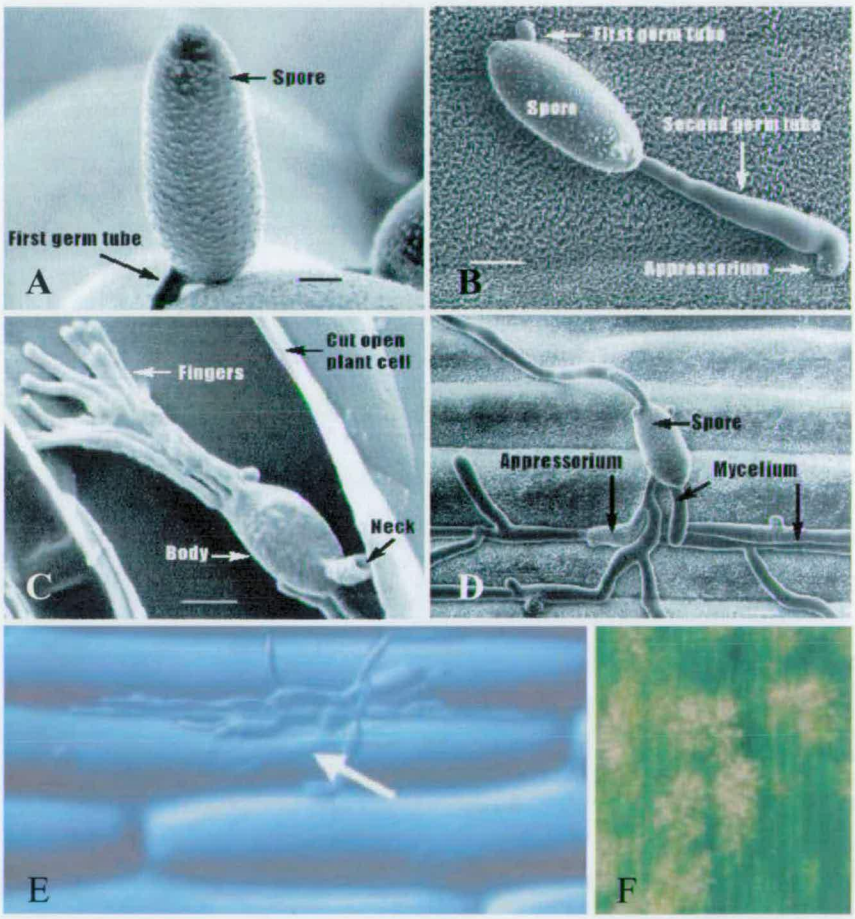


Figure 1.7. Life cycle of *Bgt* on wheat. A) Growth of primary germ tube soon after landing on the leaf surface; B) Appersorium germ tube grows upon primary germtube recognises relative compatibility; C) formation of haustoria within epidermal cell; D) a developed *Bgt* on the wheat, electron microscopic image (taken from: Carver *et al*, 2001); E) *Bgt* growth on the wheat; F) Disease symptoms (taken from:Schulze-Lefert and Vogel, 2000).

Aims of the Project

In this project, I have used the model plant, *Arabidopsis thaliana* to study its non-host resistance against the two main fungal diseases of wheat. Resistance of Arabidopsis to grey mould, *Botrytis cinerea* was also investigated. Grey mould, due to its broad spectrum of hosts, is the most common necrotroph and imperfect fungus causing fruit and general diseases on plants. Specific aims of the research investigated in this thesis were to:

- Characterize the Arabidopsis – *Blumeria graminis* f.sp. *tritici* pathosystem.
- Characterize the Arabidopsis – *Puccinia triticina* pathosystem.
- Study natural variation for nonhost resistance among the Arabidopsis accessions.
- Study the genetic basis of nonhost resistance against *Ptr* in Arabidopsis.
- Study *AS1* function in modulating plant-microbe interactions.

Chapter 2

Materials and Methods

Growth of *Arabidopsis thaliana*

Seeds were planted in a small (~ 50gr) pots followed by keeping in dark and high relative humidity (>90%) condition at 4°C for 48 hours to stimulate vernalisation. Pots were then transferred to a growth room with a short day (<10 hrs) condition until seeds germinated but were still covered, to maintain high humidity, chamber temperature ranged between 20-22°C. A week after germination, six seedlings from each genotype were transplanted to a larger pot (~150gr) as one replication. For aseptic growth, seeds were sterilised with commercial bleach for 20 minutes, washed 4 times in distilled water and maintained for 4 days in the dark at 4°C to help uniform germination. Plants were subsequently transferred to MS plates containing MS basal salts supplemented with 1% (w/v) sucrose and 1% (w/v) agar. All chemicals employed were purchased from Sigma-Aldrich UK unless stated otherwise. Petri dishes were transferred to a growth chamber with 16 hours of light at 22°C and 8 hours of dark at 18°C.

Arabidopsis genotypes

All the *Arabidopsis* accessions and the set of (Recombinant Inbred Lines) RILs derived from a Col x L*er* cross were supplied by the Nottingham Arabidopsis Stock Centre (NASC). Plants were grown under short day conditions, 9 hours light at a temperature of 20°C. Accessions tested in our experiments are listed in tables 2.1. In addition, all Arabidopsis transgenic lines and mutants used, were in a Columbia (Col) and *Landsberg erecta* (L*er*) background (Tables 2.1 and 2.2).

Table 2.1. The abbreviated name and origin of the ecotypes investigated for NHR

No	Stock No	Name	Origin	No	Stock No	Name	Origin
1	N1583	Vi-0	Germany	**41**	N1587	Wa-1	Poland
2	N1575	Uk-1	Germany	**42**	N933	Col-4	USA
3	N1307	Lc-0	UK	**43**	N1287	Kn-0	Lithuania
4	N1477	Pog-0	Canada	**44**	N1437	Oy-0	Norway
5	N1065	Can-0	Spain	**45**	N1565	Tsu-0	Japan
6	N1145	Es-0	Finland	**46**	N1265	Kas-1	India
7	N997	Bs-1	Switzerland	**47**	N949	Ang-0	Belgium
8	N1621	XXX-0	Unknown	**48**	N1337	Lip-0	Poland
9	N1567	Tu-0	Italy	**49**	N1085	Co-1	Portugal
10	N1585	Van-0	Canada	**50**	N1221	Hau-0	Denmark
11	N1615	Wü-0	Unknown	**51**	N1199	Gr-1	Austria
12	N1595	Wil-1	Russia	**52**	N1240	Is-0	Germany
13	N1227	Hi-0	Netherlands	**53**	N1218	Ha-0	Germany
14	N1553	Ts-1	Spain	**54**	N1613	Wt-5	Germany
15	N1627	Zu-0	Switzerland	**55**	N969	Bl-1	Italy
16	N1573	Ty-0	UK	**56**	N1232	Hl-3	Germany
17	N1631	Wi-0	Germany	**57**	N1226	Hi-0	Netherlands
18	N1063	Cal-0	UK	**58**	N1122	Edi-0	UK
19	N1549	Ta-0	Czechoslovakia	**59**	N1252	Jl-3	Czechoslovakia
20	N1211	Gre-0	USA	**60**	N1216	Gy-0	France
21	N1605	Wt-1	Germany	**61**	N1603	Ws-0	Russia
22	N1245	Lta-0	Morocco	**62**	N1236	Hs-0	Germany
23	N1584	Van-0	Canada	**63**	N1224	Hh-0	Germany
24	N1123	Edi-0	UK	**64**	N1154	Fe-1	Germany
25	N935	Aa-0	Germany	**65**	N1186	Ge-0	Switzerland
26	N1029	Bur-0	Ireland	**66**	N1244	Ita-0	Morocco
27	N1619	XX-0	Unknown	**67**	N1003	Bsch-0	Germany
28	N963	Bd-0	Germany	**68**	N1623	Yo-0	USA
29	N1381	Mt-0	Libya	**69**	N1605	Wt-1	Germany
30	N1073	Chi-0	Russia	**70**	N995	Br-0	Czechoslovakia
31	N1589	Wc-1	Germany	**71**	N1067	Cen-0	France
32	N1220	Hau-0	Denmark	**72**	N1040	Bu-19	Germany
33	N1115	Dr-0	Germany	**73**	N1488	Rou-0	France
34	N1117	Dra-0	Czechoslovakia	**74**	N1376	Ms-0	Russia
35	N1455	Pi-0	Austria	**75**	N1506	Sap-0	Czechoslovakia
36	N1093	Col-0	USA	**76**	N948	Ang-0	Belgium
37	N1061	Ca-0	Germany	**77**	N1514	Sav-0	Czechoslovakia
38	N1642	L*er*-1	Germany	**78**	N1238	In-0	Austria
39	N1617	X-0	Unknown	**79**	N901	Ag-0	France
40	N1369	Mh-1	Poland				

Table 2.2. Arabidopsis mutants investigated for NHR

Genotype	Phenotype	Refrence	Source
Col	Wild type		NASC
Ler	Wild type		NASC
as1 (Ler)	*Asymmetric leaves*	(Byrne *et al.*, 2000)	Hudson, Edinburgh
as1 (Col)	*Asymmetric leaves*	(Redei, 1965)	Hudson, Edinburgh
As135S:SkARP1	Wild-type	**(Harrison *et al.* 2005)**	Hudson, Edinburgh
NahG	Salicylate hydroxylase transgenic	(Lawton *et al.*, 1995)	Novartis, USA
ein2-1	Ethylene insensitive	(Guzman and Ecker, 1990)	NASC
Coi1-1	Jasmonate insensitive	(Feys *et al.*, 1994)	Turner, University of East Anglia
npr1-1	SAR insensitive	(Cao *et al.*, 1997)	Dong , Duke University
pen1	Impaired penetration resistance to *Bgh*	(Collins *et al.*, 2003)	Hans Thordal-Christensen, Risø- Denmark
pen2	Impaired penetration resistance to *Bgh*	(Lipka *et al.*, 2005)	V. Lipka , Max Planck Institute
pen3	Impaired penetration resistance to *Bgh*	(Stein *et al.*, 2006)	P. Schulze-Lefert, Max Planck Institute
rar1	Impaired *R* mediated signalling	(Muskett *et al.*, 2002)	J. Parker, Max Planck Institute
sgt1	Impaired *R* mediated signalling	(Tor *et al.*, 2002)	J. Parker, Max Planck Institute
N.sylvestris	Wild type		Koning, Connecticut, USA
AF18B.1	*Asymmetric leaves*	(Mchale and Koning, 2004)	Koning, Connecticut, USA

Pathogen growth and disease resistance assay

Wheat leaf rust, *Puccinia triticina* (*Ptr*). The *Ptr* isolate WBRS-97-3 (Zhang *et al.*, 2003) was maintained on the susceptible wheat cultivar Vuka. Briefly, 10-day-old, wheat plants, pre-sprayed with water, were dusted with rust conidia blended with talcum powder (1:3, v/v) and left overnight, in the dark, under high humidity, at 20°C, to promote spore germination. After a further 12 days, *Ptr* spores were collected from the leaf surface. Experiments were carried out in a randomized complete block design with three replications. Infection of four-week-old Arabidopsis seedlings with *Ptr* was undertaken by dusting plants, pre-sprayed with water, with a *Ptr*-talcum powder mix (1:3, v/v). Every sixty plants

were inoculated with a mixture of 15 mg of fresh *Ptr* spores in 175 mg of talcum powder. After inoculation, plants were left overnight in the dark, within a sealed humid chamber at 20°C, optimal for *Ptr* germination. Subsequently, inoculated *Arabidopsis* plants were uncovered and left for a further 7 days prior to sampling.

Wheat powdery mildew, *Blumeria graminis* f.sp. *tritici* (*Bgt*). All races of *Bgt* were obtained from Syngenta (Jealott's Hill, UK) and maintained on the wheat cultivar Hereward. The *Bgt* race routinely employed was Sav GH135 and named *Bgt1*. The other *Bgt* race was W11S, and denoted *Bgt2*. *Bgt* conidia were dust-inoculated directly on the leaf surface of wheat or Arabidopsis plants. The inoculated Arabidopsis plants were left in the growth room for 4 days before harvesting leaf samples. The percentage of haustorial formation was calculated as the number of haustoria divided by the number of germinated conidia. The epidermal cell death percentage was calculated as the number of dead cell divided by the number of germinated conidia.

***Pseudomonas syringae* pv tomato DC3000.** *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) (Whalen *et al.*, 1991) was grown in King's Broth (KB) liquid medium supplemented with 50mg/l rifampicin (KING *et al.*, 1954). Four week old plants were infected with a *Pst* DC3000 suspension in 10 mM $MgCl_2$ by completely infiltrating the abaxial side of the leaf with a 1 ml syringe (Cao *et al.*, 1994). Three leaves per plant and four plants per line were infiltrated. After three days, leaves were collected for bacterial growth analysis. Leaves from each plant were weighed and ground in 1 ml 10 mM $MgCl_2$ using a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension and 100 µl of each dilution was spread onto KB medium plates containing 50mg/l rifampicin. The plates were incubated at 30°C for two days and the number of bacterial colonies for each plate was recorded. Bacterial counts were statistically analysed using a t-test. *Pst* DC3000 isolates carrying the avirulence genes *avrB* and *avrRps4*

respectively, were grown on KB medium supplemented with 50mg/l rifampicin and 50mg/l kanamycin. Liquid cultures were grown on a shaker at 30°C and cells were pelleted by centrifugation and were re-suspended for plant inoculation in 10 mM $MgCl_2$. For inoculations, around 10µl of avirulent strain *Pst* DC3000 solution were forced under the abaxial epidermis using a 1 ml syringe. Successful inoculations were visualized by the appearance of a watery area under the epidermis. The HR was measured using an electro-conductivity assay (Tang *et.al.* 2007).

Grey mould (*Botrytis cinerea*). *Botrytis cinerea* isolated from strawberry plants were grown on oat meal medium for 7-10 days. Spores were collected by washing the colonies with sterile water, passed through a cheese cloth tissue to remove mycelium and centrifuged at 3,500×g for 10 minutes to pellet the spores. The pellet was re-suspended in 1ml of half-strength potato dextrose broth (PDB) medium, and dilutions thereafter were used to determine spore concentration using a haemocytometer chamber and a light microscope. To evaluate resistance against *B. cinerea*, four week-old *Arabidopsis* plants were sprayed with a conidial suspension containing 5 x 105 spores/ml re-suspended in ½ PDB until droplets ran off or drop inoculated by applying a 5 µl drop of spore suspension on the surface of three leaves per plant. Inoculated plants were incubated at 100% relative humidity for 4-6 days before being examined for typical necrotic lesions caused by *B. cineria* infection.

Scoring was as follows: 0 = no necrotic lesions, 1 = plants showing small-dry lesions, 2 = plants showing a mix of small and medium size lesions, 3 = plants showing medium size and spreading lesions, 4 = plants showing predominantly spreading lesions, 5 = plants showing predominantly wide necrotic lesions. Plants in different replicates were assigned a disease index as follows: $D.I.= \sum i.Xj/n$, where i = infection class, j = the number of plants scored for that infection class and n = the total number of plants in the replicate (Barathova and Betina, 1976). Twenty plants were infected for each line.

Histochemical staining

Trypan blue staining. Cells committed to die were visualized as described (Yun et al. 2003). To facilitate visibility of the pathogen structures and dead plant cells, the leaf samples were stained with lactophenol-trypan blue (TB) (glycerol, lactic acid and phenol, 250ml/l of each, 1g/l trypan blue powder) by boiling for two minutes. After cooling down to room temperature, stained leaves were cleared in chloral hydrate solution (2.5 g/l) overnight with constant shaking. The leaf samples were equilibrated in 70% (v/v) glycerol and mounted on the microscope slides (Bowling *et al.*, 1997).

Aniline blue staining. The extent of callose deposition was determined by aniline blue staining. Following vacuum infiltration and boiling in lactophenol, the leaves were incubated for 1 hour, transferred to saturated chloral hydrate and agitated overnight. Samples were viewed by epifluorescence microscopy with excitation at 430 nm (Dietrich *et al.*, 1994).

Diaminobenzidine DAB Staining. Peroxides were stained with DAB (ThordalChristensen *et al.*, 1997). Dipped leaf samples into DAB solution (0.1 g DAB in 100 ml of boiled ddH_2O) and left for overnight. The DAB solution was replaced with 96% ethanol then boiled until all the chlorophyll decoloured. Samples were cooled down and washed twice with ddH_2O. They were then mounted on a slide and investigated by light microscopy. Indistinguishable, strong H_2O_2-dependent DAB staining was observed in all the *Arabidopsis* lines described in this work. Catalase effectively eliminated the DAB stain.

DAF staining. NO accumulation was visualized with DAF-2DA (Alexis) as previously outlined (Zeidler *et al.*, 2004). For quantification of DAB staining: 10 leaves per line, per time point, from three independent experiments, were quantified using the "saturation" function in Paint Shop Pro 8. For quantification of NO accumulation, the extent of DAF-2DA staining was determined as above except that the "histogram" function in the Image J program was employed.

RNA blot Analysis

Total RNA was extracted from Arabidopsis leaves harvested from four-week old plants using the Trizol protocol (ww.science.siu.edu/nickrent/PLB420/DNA. Technique/ Trizol.method.html). In summary, leaf tissue (approximately 0.1g) was ground inside a 1.5 ml Eppendorf tube using an electric micropestle, followed by adding 200µl of Trizol solution. In sequence, tubes were vortexed vigorously until the sample was completely dispersed and 40µl chloroform added into each tube and vortexed again for another 15 seconds. After 2-3 minutes standing at room temperature, the samples were centrifuged at 10,000×g for 10 minutes and the supernatant carefully transferred into a new tube. Isopropanol and 0.8 M sodium citrate/1.2 M NaCl was added into each tube as half the volume of the aqueous phase and mixed by inverting the tubes gently. Tubes were let to stand for 10 minutes before another centrifugation cycle at 10,000×g for 10 minutes. The pellet was washed in 75% ethanol (v/v) once, followed by an extra centrifugation step at 10,000×g for 5 minutes. The pellet was dried at room temperature and then dissolved in 50 µl DEPC-treated water. Alternatively, RNA extraction was carried out using an RNA kit (Qiagen, CA, USA) according to the instructions of the supplier. The absorbance of each sample was measured at 260 nm, and used to calculate the concentration of RNA. Samples (10 µg) were separated on formaldehyde-agarose gels (Sambrook *et al.*, 1989), transferred to a Hybond™-N hybridization membrane according to the instructions of the supplier (Amersham, UK) and hybridized with the relevant probes (described below). Dextran sulphate (10% w/v) was included in the pre-hybridization / hybridization solution in order to enhance efficient binding of the probe (Sambrook *et al.*, 1989). Blots were washed twice for 30 min each at 65°C in 4 × SSC, 1% (w/v) SDS, which was followed by two washes at 65°C in 4 × SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate period. Blots were stripped by incubation in boiling 0.1% (w/v) SDS and washing in 0.5 × SSC for 30 min at room temperature, before hybridization with a subsequent probe (Sambrook *et al.*, 1989). Probes were prepared by amplification of appropriate sequences using PCR and directly

Table 2.3. DNA probes employed for Northern blot analysis

Gene	Template	Forward primer
PR-1	*TA-PR1*	CTGCAGACTCATACACTCTGG
PDF1.2	Genomic DNA	TCATGGCTAAGTTTGCTTCC
PAD3	Genomic DNA	CCGGTGAATCTTGAGAGAGCC

purified using a kit (Promega, UK). Alternatively, probes were generated from plasmids by digestion with relevant restriction enzymes and purified from the gel using a gel purification kit purchased form Qiagen (Table 2.3). Sequences for the PCR primers and templates used for each probe are reported. Probes were labelled with α-32P-dCTP by random priming using the Prime-a-Gene® labelling system (Promega, UK).

Biochemical Analyses

Salicylic acid. Free and conjugated endogenous SA levels were determined by HPLC analysis, as described (Aboul-Soud *et al.*, 2004) with minor modifications. Essentially 200 mg of leaf tissue per sample was collected and promptly frozen in liquid nitrogen. Samples were then ground in liquid nitrogen using a mortar and pestle and pulverized tissue transferred to a Falcon tube, followed by the addition of 1 ml of 90% methanol (v/v) and vortexing for 1 minute. It is important that the sample did not thaw before adding MeOH. The sample was then transferred to a 2 ml microtube, centrifuged at 15,000 ×g for 5 minutes and supernatant transferred to a new tube. The pellet was resuspended in 1 ml 100% methanol, centrifuged and the two supernatant pooled together and dried in a speed vacuum centrifuge at medium temperature. The residue resulting from drying the supernatant was then resuspended in 1 ml 5% of trichloroacetic acid, followed by the addition of 1 ml of ethyl acetate: cyclopentane: isopropanol (50:50:1) and vortexed for 1

minute. The organic phase was transferred to a new tube. The aqueous phase was re-extracted with another 1 ml of the organic 50:50:1 mix and the two supernatants pooled together and evaporated under heat in the vacuum centrifuge. The aqueous phase was then acidified to pH 1, boiled for half hour to release conjugated SA and extracted with the organic mix twice. The two supernatants were pooled together and dried in the vacuum centrifuge. The residues were dissolved in 200 µl of 50% methanol (v/v), filtered and subjected to High Performance Liquid Chromatography (HPLC) analysis.

Camalexin. Camalexin was extracted and measured by HPLC as outlined in a preceding study (Glazebrook and Ausubel, 1994). Briefly, leaf disks (50 mg FW) were incubated in 1.7 mL of methanol (80%) at 65°C for 20 min. Leaf tissue was removed, and methanol extracts subjected to evaporation under reduced pressure (speed vacuum) until the volume was reduced to approximately 500 µL. Samples were 48 extracted with 2x 150µL of chloroform, and the chloroform phases (the green lower phases) removed into clean tubes. Chloroform was then evaporated under reduced pressure and residues dissolved in 200 µL of 50% methanol. After filtration, samples were quantified by using HPLC with a Dionex HPLC system equipped with AS50 autosampler, AS50 thermal compartment, GS50 gradient pump, PDA-100UV-is detector and RF2000 fluorescence detector. The analytical column used was a 250 x 4.6mm Phenomenex Luna 5µ C-18 (2) and a variable wavelength spectrofluorometric detector (excitation 318 nm/emission 385 nm) was used. Quantification was performed using chromaleon chromatography software by integration of peak area and a calibration curve of camalexin standards. The eluents were used are as follow: 1. at time 0 - 5 min 60% MeOH to 70% MeOH; 2. time 5-10 min Isocratic 70% MeOH and flow rate 1 ml/min at column temperature of 35 degrees Celsius. Camalexin was quantified by comparing peak areas with camalexin standards (1µg/ml) (provided by Dr Jane Glazebrook, University of Minnesota-Twin cities, MN, USA) with respect to retention time (9 min) and UV absorption spectra.

Mapping using SSLP markers

F2 plants expressing the desired phenotype were selected for mapping, which was performed using the simple sequence-length polymorphisms (SSLPs) protocol (Bell and Ecker, 1994). This approach is based on PCR of polymorphic genomic DNA sequences between different accessions. The PCR primers used for SSLP markers are outlined at The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/). Genomic DNA was extracted from leaf tissue using a CTAB DNA extraction protocol (Dellaporta, 1983). Approximately, 3 leaves from each plant were ground in 0.4 ml DNA extraction buffer with 0.2% 2-mercaptoethanol. Samples were incubated at 60°C for 30 min followed by extraction with an equal volume of chloroform. After centrifugation (6,000 ×g, 15 min), the DNA was precipitated from the resulting supernatant by the addition of an equal volume of isopropanol. After a further centrifugation step (6,000 ×g, 20 min), the DNA pellet was washed in 70% (v/v) ethanol and finally re-suspended in 60 µl of water. Primers for the different markers are listed in Table 2.4 and all primers were manufactured by Sigma (UK). PCR reactions were performed using 10× buffer, magnesium chloride and *Taq* polymerase and deoxynucleotides from Promega (UK). The PCR reactions were carried out in 20 µl volume containing 1-2 µl of the DNA miniprep (approximately 10-20 ng DNA), 5 pmol of each primer, 200 µM each of four deoxynucleotides, 2mM magnesium chloride and 1 unit of *Taq* polymerase. The PCR reactions were carried out in a PTC-200 Peltier thermal cycler. The PCR conditions were as follows: 30 s at 94°C, primer annealing for 30 s at 56°C, primer elongation for 1 min. This cycle was repeated 30 times. 4 µl of 6× loading dye (Sambrook *et al.* 1989) was added into each tube of the SSLP PCR products prior to electrophoresis. Ws-1, Col-0 and/or L*er* samples were included on each gel as controls and run in adjacent lanes in order to visualize polymorphisms. A 1 kb and/or 100 bp DNA ladder (New England Biolabs) were also included on each gel in order to determine the size of different PCR or restriction digestion products.

Table 2.4. List of CAPS and SSLP markers used in mapping experiments. Restriction enzymes required for analysis of CAPS markers, gel conditions for analysis of PCR products, observed PCR products polymorphic between Col-0 and L*er* and primer sequences for the markers are shown. [a]cM represents centimorgans.* MP represents MetaPhor agarose gel.

Chr	cM [a]	Name	Type	Enzyme	Gel	Fragment Col-0 (kb)	Ler (kb)	Forward primer seq	Reverse primer seq
1	9.3	Nga63	SSLP		2% 2h,82V	0.111	0.089	ACCCAAGTGATCGCCACC	AACCAAGGCACAGAAGCG
1	46	AthSO392	SSLP		3% MP* 4.5h ,45V	0.142	0.156	TTTGGAGTTAGACACGGATCTG	GTTGATCGCAGCTTGATAAGC
1	78	F6D8	SSLP		2% 2h,82V	0.321	0.226	TTCGTGAGGTTATGCCGATC	TTGACTCCGTTCCAGAGT
1	83.32	Nga128	SSLP		2% 2h,82V	0.18	0.19	GGTCTGTTGATGTCGTAAGTCG	ATCTTGAAACCCTTTAGGGAGGG
1	83.83	Nga280	SSLP		3% MP* 4.5h ,45V	0.105	0.085	GGCTCCATAAAAAGTGCACC	CTGATCTCACGGACAATAGTGC
1	~84	T6H22	INS/DEL		3% MP* 4.5h ,45V	0.263	0.252	TCCAATATGCTTAGCTCTGG	AGCCAAATCAATGCAGTCTC
1	~84	T30E16-2	INS/DEL		3% MP* 4.5h ,45V	0.365	0.360	CGTAGTAGAGTCTTAGCAGTG	CCGATTTGCTACAGTGACAA
1	~84	T30E16	INS/DEL		3% MP* 4.5h ,45V	0.221	0.278	GCTTTAGGAGCCCGTGAATC	CACCCATGCAAGTGAATCAG
1	85	F8A5	INS/DEL		2% 2h,82V	0.235	0.177	ACGCTCGTTAGGTACGCCT	GGAGTGAGTAACGTGCATG
1	88	F2401	INS/DEL		3% MP* 4.5h ,45V	0.221	0.201	GCAGCCATCAACATTGTTGA	ACACTCTCGACACATTGGCA
1	93	F5I14	INS/DEL		3% MP* 4.5h ,45V	0.236	0.181	GAGAAGATGAAGGCCTATGTG	CCCTCATATGGTCAGTCAACA
1	115.5	Nga111	SSLP		3% MP* 4.5h ,45V	0.128	0.162	TGTTTTTTAGGACAAATGGCG	CTCCAGTTGGAAGCTAAAGGG
2	30	THY1	CAPS	RSA1	2% 2h,82V	0.81	0.6/0.1	0.6/0.5/0.4/0.3/0.1	GGCGACCTTGGACCTGTATACG	AACCGCCATTTTCATTTCTATC
2	50.65	Nga1126	SSLP		3% MP* 4.5h ,45V	0.191	0.199	GCACAGTCCAAGTCACAACC	CGCTACGCTTTTCGGTAAAG
2	73	Nga168	SSLP		3% MP* 4.5h ,45V	0.151	0.135	GAGGACATGTATAGGAGCCTCG	TCGTCTACTGCACTGCCG
3	16.35	Nga126	SSLP		2% 2h,82V	0.119	0.147	CAAGAGCAATATCAAGAGCAGC	GAAAAAACGCTACTTTCGTGG
3	20.56	Nga162	SSLP		3% MP* 4.5h ,45V	0.107	0.089	CTCTGTCACTCTTTTCCTCTGG	CATCGAATTTGCATCTGAGG
3	75	Nga707	SSLP		3% MP* 4.5h ,45V	0.132	0.128	TGAATGCGTCCAGTGAGAAG	CTCTCTGCCTCTCGCTGG
4	22.92	Nga12	SSLP		2% 2h,82V	0.247	0.234	TGATGCTCTCTGAAACAAGAGC	AATGTTGTCCTCCCCTCCTC
4	29.6	Nga1111	SSLP		4% MP* 6.5h, 45V	0.148	0.154	AGTTCCAGATTGAGCTTTGAGC	GGGTTCGGTTACAATCGTGT
4	57.6	G4539	CAPS	HindIII	1% 2h, 82V	0.6	0.6	0.48/0.12	GGTCATCCGTTCCCAGGTAAAG	GGACGTAGAATCTGAGAGCTC
4	63.16	AG	CAPS	XBAI	1% 2h, 82V	1.366	1.366	1.073;0.293	CAAACACCATTTAATCTTGACA	CAACAGGTTTCTTCTTCTTCTC
4	83.41	Nga1139	SSLP		3% MP* 4.5h ,45V	0.114	0.118	TTTTTCCTTGTGTTGCATTCC	TAGCCGGATGAGTTGGTACC
4	75.7	RPS2	CAPS	Sau3A	2% 2h,82V	0.785	0.18/0.605	0.18/0.251/0.354	CTCAGAGTCTTGGACTTGTCG	TTCGACGGATGGACTCTCGTG
4	102	Nga1107	SSLP		2% 2h,82V	0.15	0.14	GCGAAAAAACAAAAAAATCCA	CGACGAATCGACAGAATTAGG
5	23.72	Nga249	SSLP		2% 2h,82V	0.125	0.115	GGATCCCTAACTGTAAAATCCC	TACCGTCAATTTCATCGCC
5	62	AtSO262	SSLP		3% MP* 4.5h ,45V	0.145	0.159	CTCCACCAATCATGATGCAAATG	TGATGTTGATGGAGATGGTCA
5	103	AthSO191	SSLP		3% MP* 4.5h ,45V	0.148	0.156	TGATGTTGATGGAGATGGTCA	CTCCACCAATCATGCAAATG

Laser scanning confocal microscopy

Confocal images were obtained using a BioRad Radiance 2100 system mounted on a Nikon Eclipse TE300 inverted microscope. Arabidopsis-*Ptr* interactions were imaged with a 40x dry objective, with 488 nm excitation from an argon ion laser and a combination of filters (Dichronis filter 560DCL PXP, Blocking filter HQ500LP, Emission filter HQ530SP).

Arabidopsis leaves infected with *Bgt1* were cleared and stained with Alexa fluor 488 as previously described (Duncan and Howard, 2000). Samples were imaged using dry ×20 and ×60 water immersion plan apo objectives, with 488 nm excitation from an argon ion laser. Alexa fluor 488 was obtained from Molecular Probes (Eugene, OR, USA).

NO imaging was undertaken using the same microscope and excitation wave length as used above. Emissions were collected at 515 (DAF-2DA) and 590 nm (chlorophyll auto fluorescence).

GFP fluorescence for AS1 was excited by an argon ion laser at 488 nm, and the fluorescence emission was detected between 505 and 530 nm. DAPI fluorescence was excited by blue diode laser at 405nm and detected between 480 to 500 nm. Nuclei stained with 1μg/ml DAPI (Theodoris *et al.*, 2003).

Data analysis and QTL identification

Data was collected from TB stained leaves by using an Olympus CX21 light microscope. To comply with the requirements of the statistical analysis, logarithmic transformation was used to improve the normal distribution of observation and homogeneity of variance (Miller, 1997). Analysis of variance was utilized to determine significant differences and Duncan's test was used for mean comparisons. Differences were accepted at the 95% significance level.

Minitab Statistical Software version 14.1 was used for the statistical analysis and Windows QTL Cartographer Version 2.5 (Wang et al., 2005) for QTL mapping.

Chapter 3

Natural Diversity of NHR in the Arabidopsis - wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*) interaction

Introduction

Pathogens clearly play an evolutionary role in shaping genetic structure of plant populations. The evolutionary dynamics of disease resistance influences both natural populations (geographical accessions) and the crops that are developed through breeding practices. Pathogens enforce a selection pressure that facilitates establishment of plant mutants to dominate the ecosystem (Rausher, 2001). The level of changes in genetic architecture of a population can vary from small to considerable depending on the destruction of the pathogen over time and space (Smith *et al.*, 2003). Natural populations contain alleles that have been selected during a very long term evolutionary process. Therefore, they are likely to represent the best characterized and adapted change of function alleles for different geographic zones (Winn, 2004). Thus the large effect of resistance genes in a species can be investigated in the individual plant populations or geographic accessions due to the allelic variation. The gradual selection process increases the probabilities that the phenotypes being affected in a quantitative fashion as subtle and little variation occur toward adaptation and only in exceptional events selection was made for alleles with major effects (Winn, 2004). Even though great differences between phenotypes are more likely due to the allelic variation at several loci (Weigel and Nordborg, 2005).

Natural variation as a key feature of evolutionary biology is also attracting interest from functional biology. In plant-pathogen interactions, plant specificity and pathogen virulence continually adapt in response to each other and has been called the co-evolutionary arms-race model (Dawkins and Krebs, 1979). The

arms-race model anticipates that high selection pressure of disease reduces tha number of *R* gene alleles by replacing old *R* genes with new and monomorphic ones (Bergelson *et al.*, 2001). In contrast, recent studies showed that for each ancient *R* gene there are many segregating alleles available (Parniske *et al.*, 1997; Stahl *et al.*, 1999; Isidore *et al.*, 2005). For instance large numbers of polymorphisms in *RPP1*, *RPP13* and *RPS2* alleles, indicates these genes are ancient and they have been present before pathogen specificity arose (Bergelson *et al.*, 2001). Therefore the balancing selection model was proposed, where alleles at *R* and *AVR* loci are maintained based on recycling relatively old genes (Holub, 2001). This model best fitted and is consistent with the guard hypothesis model for function of resistance proteins (van der Hoorn *et al.*, 2002).

Arabidopsis is well established as a model system for genetics and molecular studies and has been extremely helpful in studying plant-pathogen interactions. Arabidopsis is perfectly suited for the study of natural variation. The reasons are the small amount of heterozygosity exists in natural populations due to its self pollinating nature, has a relatively simple genetics and more importantly it has been fully sequenced (Arabidopsis, 2000). Moreover, Arabidopsis is a cosmopolitan species as stocks from many regions are readily available. Since several Recombinant Inbred Lines (RIL) were developed, positional cloning of quantitative trait loci (QTL) should become much more feasible.

The first aim of the research described in this chapter was to characterize the cellular responses of Arabidopsis to wheat powdery mildew, *Blumeria graminis* f.sp. *tritici* (*Bgt*). The second was to analysie the natural variation of nonhost resistance to *Bgt* by screening a series of Arabidopsis geographical accessions. *Bgt* is an obligate biotrophic pathogen of wheat. This pathogen is known to establish a complex and close association with its host (Jorgensen, 1988).

Results

Interaction of Arabidopsis and Wheat powdery mildew

The non-host pathogen, *Bgt* is not able to complete its life cycle on Arabidopsis plants. However, it can attach, recognise primary compatibility and develop some infection structures towards its establishment. NHR in this pathosystem is predominantly expressed before pathogen penetration. The powdery mildew attack rapidly triggers immediate induction of defence responses in Arabidopsis such as ROI accumulation, salicylic acid production, callose deposition or expression of *PR1* and *GST1* on wild type plants (Yun *et al.*, 2003).

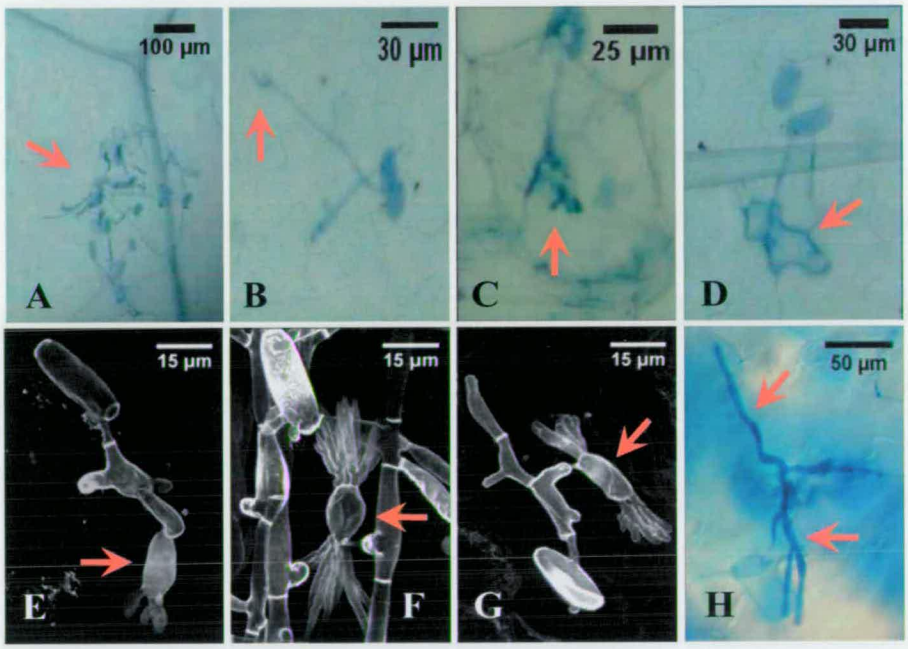


Fig 3.1. Developmental stages of *Bgt* on Arabidopsis. A) Group of germinated conidia (red arrow); B) Appressorium germ tube of *Bgt* that was precluded before any penetration attempt (red arrow); C) Successful penetration led to a premature *Bgt* haustoria (red arrow shows haustoria); D) Penetration halted by HR reaction (depicted by red arrow); E) Unilateral haustoria structure in Col-0 (shown by red arrow); F) Bilateral and fully developed haustoria in wheat (red arrow); G) Bilateral haustoria in *eds1* mutant (red arrow); H) Development of secondary hyphae on the leaf surface following haustorium formation (arrows show branches of secondary hyphae); A,B,C,D,I Trypan blue staining; E,F,G laser scanning images. Images E-G taken from Yun *et al.*, 2003.

In our experiments, conidia of *Bgt* (race Sav GH135) were dusted on the leaves of 4-week old seedlings of Arabidopsis. In general, subsequent to germination of primary germ tube (PGT) on the leaf surface, a specialized infection structure, the appressorium (Fig.3.1A-B) grows to find a suitable penetration site. Soon after locating an epidermal cell, a penetration peg emerges from appressorium and attempts to penetrate. Successful penetration may result in formation of a haustorium (Fig 3.1C), a specialized structure that uptakes nutrients and suppresses defence responses in plant (Fernandez and Heath, 1991; Hall and Williams, 2000). In case of *Bgt*-Arabidopsis, most of the attempt penetrations induce HR (Fig 3.1D). Formation of haustoria is a very low frequency phenomenon in this nonhost interaction. In most cases the formed haustoria on Arabidopsis are unilateral structure (Fig 3.1E) while in compatible interaction with wheat, haustoria forms a bilateral structure (Fig 3.1F). This infectious organ on *eds1* mutant plant is also bilateral, however, it is less developed compared to the haustoria on wheat (Fig 3.1G). A partial compatibility of *eds1* mutant to *Bgt* has been reported previously (Yun *et al.*, 2003). Then, the initiated haustoria supports formation of secondary hyphae on the leaf surface (Fig 3.1H). On wheat plants secondary hyphae initiates a network of hyphae. Conidiophores subsequently emerge from these established networks which grow to produce chains of conidia. This stage is named sporulation and is associated with visible disease symptoms. We used these marked morphological developmental stages to screen the compatibility of the Arabidopsis accessions to *Bgt*.

Screening Arabidopsis accessions

Four days post infection (dpi) of the Arabidopsis plants, marked leaves were detached and stained with trypan blue. Trypan blue staining facilitates visibility of dead cells and fungal structures. Due to NHR, most appressoria failed to penetrate into the epidermal cells. However, formation of appressorium germ

tubes (AGT) was highly frequent (Fig 3.1B). With lower frequencies, immature haustoria (Fig 3.1C) and hypersensitivity responses (HR) (Fig 3.1D) were observed.

We scored frequencies of "Appressorium formation (APF)", "HR events (HR)" and "haustorium formation (HAF)" as a percentage of total conidia landed on the leaf surface of the Arabidopsis seedlings. The experiment was carried out in a Randomised Complete Block (RCB) design with three replications to minimise the effect of environmental factors on the *Bgt*-Arabidopsis interaction. Data for HR were transformed by a $(10x + 0.5)^{1/2}$ equation for normalisation purposes (Miller, 1997). Distribution of APF was not significantly different from normal distribution so the raw data used for further statistical analysis. HAF happened in very low frequencies and did not fit into any known distribution pattern, nevertheless several different methods applied in order to normalise the distribution of collected data. Therefore the significant differences observed as a result of ANOVA analysis may not be reliable for HAF data, however, these data still can be exploited for paired comparison between two accessions in a t-test.

Table 3.1. Analysis of variance for each characteristic

S.O.V.	df	APF	HR	HAF
Replication	**2**	**6.09 ns**	**11.2 ns**	**14.77 ns**
Genotypes	**70**	**2530.17 ns**	**6363.19 *****	**1357.06 *****
Error	**140**	**5353.97**	**1529.24**	**577.39**

ns: Not significant *: significant at 5% *: significant at 0.1%**
(Data presented in the table are sum of squares),**APF:** Appressorium formation, **HR:** HR events, **HAF:** haustorium formation, **S.O.V.:** Source of variation, **df:** Degree of freedom.

Variation due to interactions of genotypes and the environment represented as replications. The replications were not significantly varied for all of the studied traits (table 3.1). In the other word, small heterogeneities in the growth chambers did not have adverse effects the on *Bgt*-Arabidopsis interaction. There was also a non-significant variability among accessions for recognising primary compatibility which led to the germination of an appressorium germ tube (AGT) and development of an infection structure (Table 3.1). On average 88.85% of conidia initiated AGT on accessions. The minimum percentage of AGT was 78.5% and the maximum was 96.58%, however, all the observed variation was due to the reasons other than plants genetic background, probably due to the heterogeneities in inocula. Apparently formation of AGTs was not subject to an inhibition by plants and the invariable formation of AGTs may point to the compatibility of the *Bgt* on Arabidopsis up to the penetration stage. On the other hand, HR and formation of haustoria did significantly change between genotypes (table 3.1).

On average, 4.48% of conidia attempting penetration encountered HR in accessions (Fig 3.2A). A minimum of 0.68% HR events were observed on Wil-1 and a maximum of 32% on Wc-1. The average number of formed haustoria was dramatically lower than HR frequencies (Fig 3.2B). The lowest percentage of haustoria was observed on Wa-1 and Oy-0 with 0.029%. In contrast the highest (4.72%) was observed on Wc-1. Despite the significant variation for haustoria among accessions, with the exception of Wc-1, the others did not exhibit a significant haustorial formation.

To study the existence of any possible relationship between these responses, Pearson correlation analysis was performed on the least-square means averages for each pair of characteristics. Correlation analysis revealed a significant

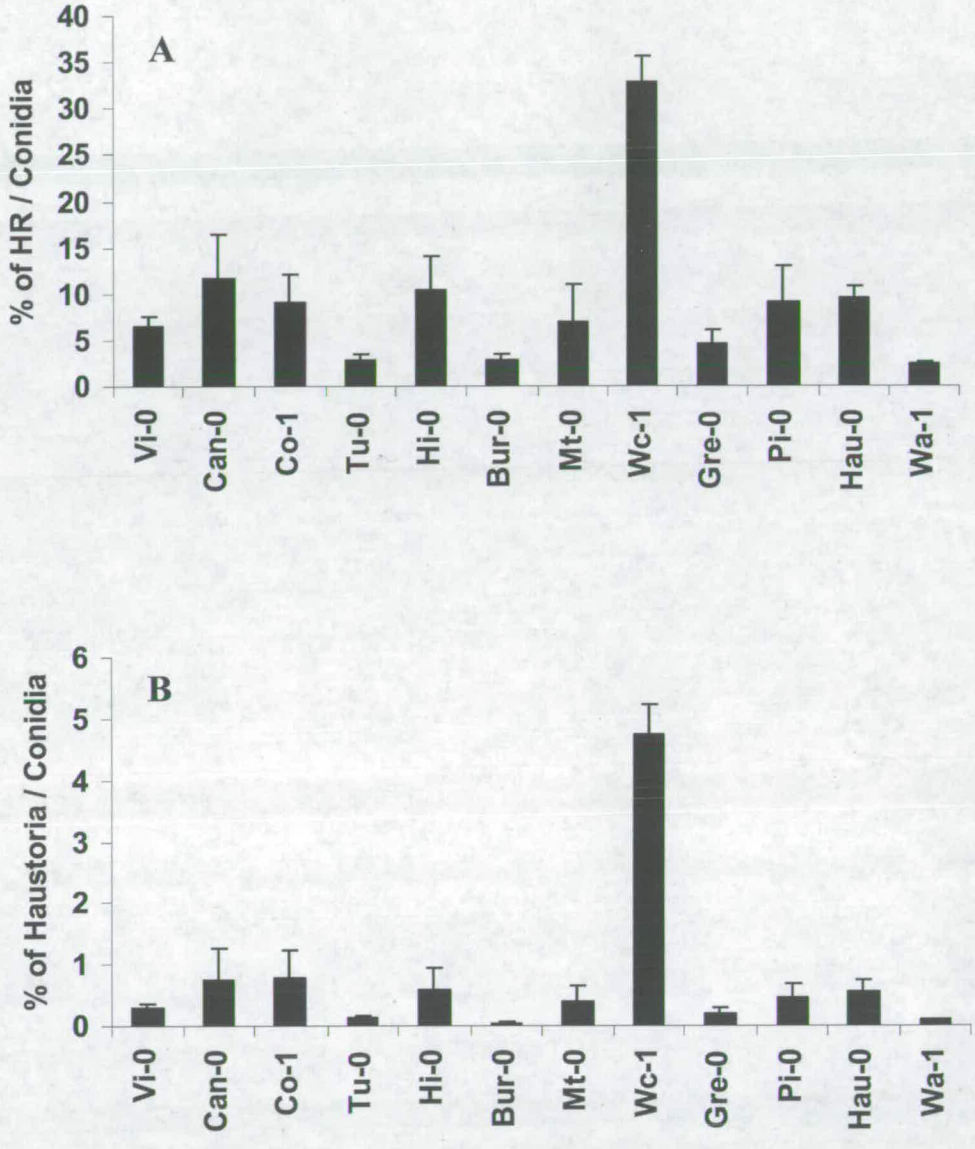


Figure 3.2. Variation of NHR among subset of the accessions challenged with *Bgt*. A) Frequency of HR events; B) Haustoria formation on accessions in response to *Bgt*; Data represents average of three replications. Error bars represent SEM.

correlation between the number of HR and HAF among the accessions (r =0.91, P< 0.001). This correlation was in a positive direction where increased frequency of haustoria would also raise the number of HR. In contrast, higher or lower number of appressorial germ tubes had no significant effects on the number of HR events and haustorium formation. The correlation between HR and APF was -0.084 (P<0.224) and between HAF and APF was -0.022 (P< 0.754) suggesting HR events or formation of haustoria was likely to be dependent on plant genotypes rather than number of formed appressoria. This may also suggest that a systemic immunity was induced in plants to protect them from further pathogen penetrations.

Mean comparisons for HR events or HAF is well applicable to determine the best parents for studying the genetic basis of each trait prior to pathogen establishment. However it cannot provide information for the best compatibility of individual accession to *Bgt* as it only takes into account one dimension of the differences. Therefore a cluster analysis was made based on the overall effects of both characteristics to identify which accession or group of accessions showed the best versus least compatibility to *Bgt*. Cluster analysis was done with Ward method using Squared Euclidean Distance (Lebeda and Jendrulek, 1987). Our analysis showed that the accessions grouped into three distinct classes (Fig 3.3); one that included some of the completely incompatible accession (group A), those with partially compatibility (group B), and a third with relatively good compatibility to *Bgt* and which had the highest dissimilarities to the other groups (Group C). The majority of accessions were placed in group A with more than 95% similarity for their resistance responses. Genotypes in this class expressed 3.6% of HR and only 0.14% of the conidia were able to successfully penetrate onto the epidermal and initiate a haustoria. The HR among the individuals of this group ranged between 0.68-7.02% while HAF varied from 0.03 to 0.38%. Five accessions formed group B. This group had 65% similarity to the first group. The average of HR and HAF in this group was 9.97% and 0.62% respectively. HR

ranged from 9.02 to 11.68% while HAFs varied from 0.44% to 0.8% within group B accessions. It seems that the higher frequencies of HR were the main basis for the differentiation of these five accessions from group A. Although, the number of observed haustoria doubled in this class, still it was very low and the divergence was negligible. The third group was highly different from the other two. This class contained only one member called Wc-1. On Wc-1 around 4.72% of appressorium were able to form haustoria and 32% of the attempt penetrations precluded pathogen development by an HR reaction.

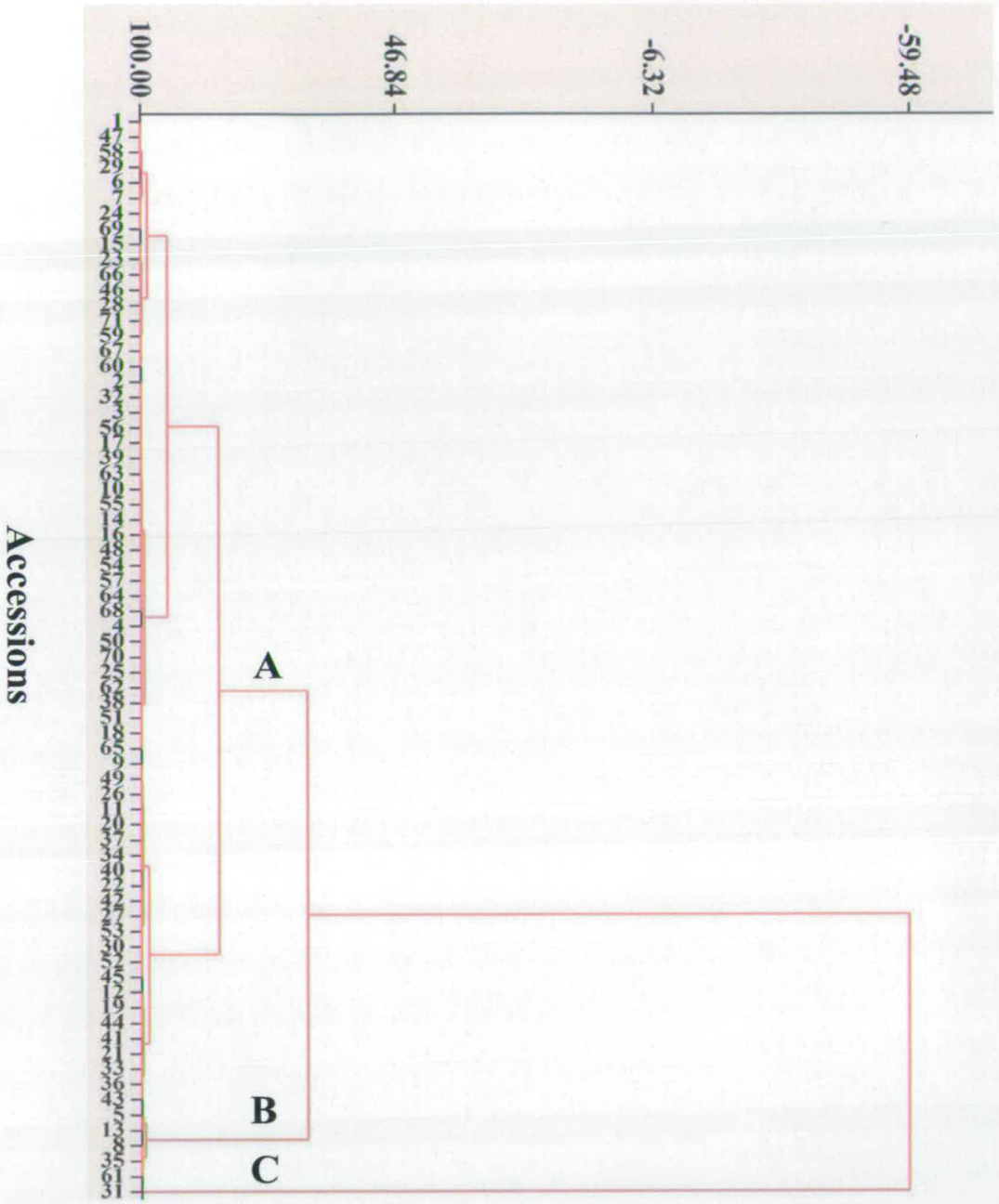


Figure 3.3. Classification of the accessions based on their response to *Bgt*. A) This class includes vast majority of accessions with more than 95% similarity; B) There are five accessions in this class that showed 64% similarity to the previous class; C) this class contains only Wa-1(Accession 31) with highest dissimilarity to the other two classes. Dendrogram drawn based on Ward Linkage method and Squared Euclidean Distance. List of accessions in this dendrogram is in the same order as listed in the Material and Methods chapter.

Characterisation of Wc-1

The origin of Wc-1 was Germany, from a region called Westercelle. This accession has long narrow leaves with serrated margins and long petioles. The average height of plant ranges from 30 to 40 cm, and its inflorescence exhibiting upright flowers and a long pedicel in a cluster (Figure 3.4A-B). Wc-1 can grow at altitudes between 1 and 100 m, a latitude of N53/N53 and a longitude of E10/E10 (NASC webpage). This accession showed a relatively higher penetration of wheat powdery mildew which was the highest amongst the accessions we tested. Higher frequencies of haustorial formation on this accession supported enhanced development of secondary hyphae and an increased number of HR responses (Fig 3.4C-D). Aniline blue staining revealed that callose deposition occurred as a resistance response around epidermal cells (Fig 3.4E-F). Callose is a (1-3) β-D-glucan, which sometimes is deposited on cell walls as a defence response for strengthening purposes upon microbial attack (Bélanger and Bushnell, 2002).

Haustoria in Wc-1 are able to develop into a bilateral structure similar to those observed in *eds-1* and the host plant (wheat) (Fig 3.4G-H). Along with its bilateral structure, each of its branches forms several fingers (Fig 3.4I). However, like *eds1*, haustoria in Wc-1 are not as developed as those seen on wheat plants. Moreover, in Wc-1 callose deposition surrounds haustorial structures (Fig 3.4G). This type of plant defence response effectively breaks up any connection between the plant and powdery mildew. The callose deposition occurred jointly with epidermal cell death.

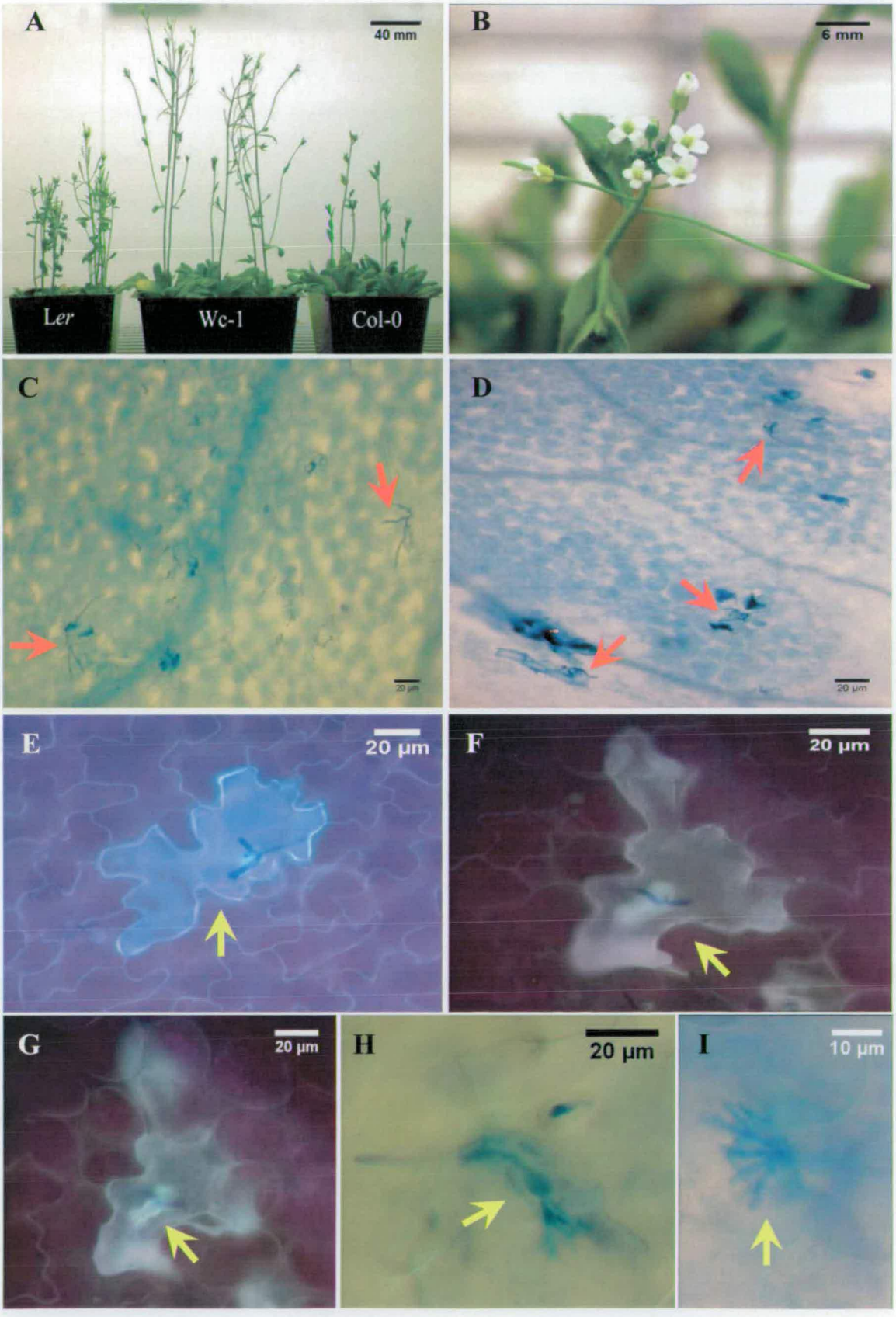

A
40 mm
Ler
Wc-1
Col-0
B
6 mm
C
D
E
20 µm
F
20 µm
G
20 µm
H
20 µm
I
10 µm

Figure 3.4. Botanical features and development of *Bgt* on Wc-1. A) Height and earliness of Wc-1 compare to well-known wild types; Col-0 and L*er*; B) Structure of flower inflorescence in Wc-1, longer petioles along with less dense flowering cluster compare to wild types; C) Broad spectrum image of *Bgt* interaction on Wc-1, arrows shows secondary hyphae which are more developed than the one on wild types; D) Another overview of the powdery mildew infection on Wc-1, arrows points to the development of secondary hyphae which all arrested with HR reaction; In both images of C and D, the number of successful penetrations, late halted pathogen growth and HRs are considerable; E and F) HR response on challenged Wc-1 with *Bgt*, arrows depicted the outlier callose deposition in the invaded epidermal cells; G) Encased haustorium with callose in Wc-1 which was associated with epidermal cell death arrow points to the haustorium; H) Bilateral structure of haustorium envaginated in epidermis of Wc-1; I) Fingers developed on one of the branches of *Bgt* haustorium in Wc-1. Images C,D,H,I prepared through trypan staining and visualised with light microscope; Images E,F,G stained via aniline blue method and visualised with epiflorescence microscopic technique.

To evaluate this partial compatibility, crosses were made between Wc-1 and Col-0, which exhibiting strong resistance against *Bgt*. Furthermore, Wc-1 was crossed with the *eds-1* mutant. HR events were decreased in F1's of both crosses relative to Wc-1 (Fig. 3.5 A,C). The Col-0 plants exhibit a very effective pre-penetration non-host resistance, whereas development of *Bgt* is restricted on *eds1* after penetration (Yun *et al.,* 2003). The HAF observed on F1 plants from both crosses was not similar to Wc-1 or *eds-1* (Fig 3.5B,D). These results again prompted the hypothesis that recessive gene(s) are supporting the higher frequencies of penetration that may only be revealed by studying the F2 generation. On the other hand, as *Bgt* was able to form haustoria with relatively high frequencies on both Wc-1 and *eds1*, observing enhanced resistance in plants was an indication of the possibility that different gene(s) underpinned penetration in Wc-1 and *eds1*. The presence of transgressive segregation among F2 populations of Wc-1 and *eds1* suggests a potentially different mechanism for the lack of penetration resistance in these genotypes.

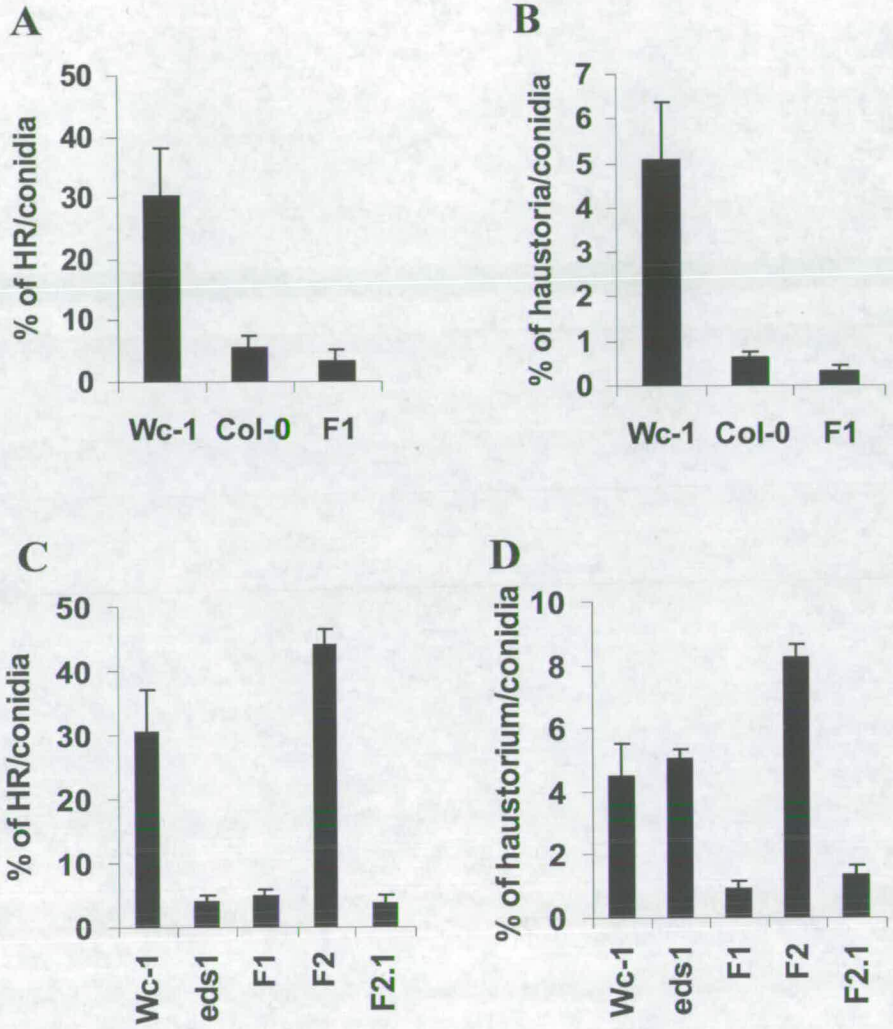


Figure 3.5. Inheritance of haustoria formation and HR events in Wc-1. A) Frequency of HR events was also reduced in F1 plants; B) Similarly, haustoria formation in F1 of Col-0 and Wa-1 reduced to similar level of Col-0;; C) HR events was reduced in F1 of *eds1* and Wa-1, also transgressive segregation was obvious among F2 population; D) Interestingly a higher frequency of haustorial formation in both *eds1* and Wa-1 was not supported in their F1 plants. However, in F2 transgressive segregation observed toward both higher and lower HAF; Data represents averages of four replications of parental lines and F1 plants. The average of four leaves from each F2 plants is presented here. Error bars represent standard errors.

The role of cell polarization and rearrangement of the cytoskeleton was demonstrated at the penetration site previously (Yun *et al.*, 2003; Shimada *et al.*, 2006). Treatment of *eds1* mutants with 2µg ml^{-1} cytochalasin E (CE) prior to inoculation with powdery mildew supported significant growth of secondary hyphae in this nonhost interaction. The increased number of penetrations on *eds1* due to application of CE accompanied with the weakened basal resistance in *eds1* and significantly compromised NHR in response to *Bgt* (Yun *et al.*, 2003). Thus we used the same pharmacological approach as Yun *et al.* 2003, to inhibit actin polymerisation in Wc-1. Likewise, loss of cytoskeletal function in Wc-1 resulted in an increased frequency of penetration up to 8.46%, relative to 4.75% of haustorial formation in *Bgt* challenged Wc-1 without CE treatment. This indicated that precluding vesicle trafficking temporarily allowed more *Bgt* penetration similar to *eds1*; however, resistance was restored more quickly in Wc-1. In contrast to *eds1*, haustoria of wheat powdery mildew on Wc-1 were not able to support growth of secondary hyphae. However, there were higher amounts of HR observed in the CE treated Wc-1 plants following inoculation with *Bgt*.

Discussion

NHR provides defence mechanisms against the majority of pathogens. Multifactorial responses in NHR imply this type of resistance might have a quantitative genetic basis, in that many genes are involved in the mediation of this response (Heath, 1996). Recent research on NHR raised new insights and identified several genes and components involved in NHR (Ellis, 2006; Nurnberger and Lipka, 2005). However, a theory assumes that NHR is a consequence of an evolutionary path resulting from co-variation of plant fitness and disease resistance (Heath, 1991). It was also demonstrated that nearly all plant traits are heritable under some environmental conditions and natural selection does not apply strict and high selection pressure on quantitative traits (Winn, 2004; Kingsolver *et al.*, 2001). This may provide a suitable ground for

major variability between geographic accessions. It also highlights the importance of natural diversity as a complementary approach to uncover features of NHR, which may have been overlooked in the study of individual mutants.

Inoculation of Arabidopsis accessions with wheat powdery mildew triggered an invariable resistance in the vast majority of accessions which was early and highly effective. On the other hand, in terms of penetration rate and the number of HR events, NHR was relatively compromised in some of the accessions. The highest numbers of penetration and haustorial structures were observed in Wc-1, which supported limited development of secondary hyphae. However, the secondary hyphae itself was not able to support further penetration of the epidermal cells and initiation of supplementary haustoria. This is a phenomenon that normally happens on host plants in order to support extensive development of a secondary hyphae network. Lack of further penetration may indirectly indicate the involvement of a systemic immunity which was accompanied with the improvement of the initial defect of cell wall penetration resistance. As it was shown previously, systemic immunity is induced through a conserved signalling pathway which involves salicylic acid and jasmonates (Truman *et al.,* 2007; Grant and Lamb, 2006). In this regard, participation of two major resistance pathways prompt the hypothesis that even effectors secreted during pre-penetration stage might trigger systemic immunity. The similarity of NHR responses among substantial numbers of accessions against *Bgt* supports the hypothesis that plants possibly have evolved detection systems for multiple and highly invariant PAMPs which trigger non-host defence (Abramovitch *et al.*, 2006). Thus, wheat powdery mildew probably secretes pre-haustorial effector molecules into Arabidopsis epidermal cells. As pathogen effectors are likely to be host specific these effectors might fail to function in Arabidopsis. Hence, those inactive effectors might trigger resistance responses in the majority of accessions. To confirm this idea, identification of few wheat powdery mildew effectors is necessary.

Three lab mutants of Arabidopsis were identified which allowed at least five-fold higher *Bgh* penetration compared to the wild type. Furthermore, *Bgh* interaction

with these mutants resulted in a significantly increased number of HR-like cell death events (Collins *et al.*, 2003; Lipka *et al.*, 2005; Stein *et al.*, 2006). Likewise a high incidence of wheat powdery mildew penetration was also observed on *pen1* and *pen2* (Kang J, personal communication). Similar to *pen* mutants, the Wc-1 accession was mostly compromised in penetration resistance but did not support extensive development of secondary hyphae. A higher rate of penetration in Wc-1 triggered a significantly enhanced number of hypersensitivity responses relative to the other accessions. Therefore, lack of penetration resistance in Wc-1 morphologically can be comparable to those *pen* mutants, as a naturally occurring cell wall penetration (*pen)* gene which was inherited in a recessive fashion.

A remarkable number of HR events were observed in the Wc-1 following *Bgt* inoculation. The HR is considered as a marked resistance response of plants to the invading pathogen and as an indicator of *R* gene responses (Xiao *et al.*, 1997). However, the extent to which gene-for-gene applies to nonhost interactions is still unclear. There are assumptions that differentiate molecular mechanisms for expression of hypersensitivity response toward host and nonhost pathogens interaction (Kumudini *et al.*, 2001). A major difference was highlighted regarding the formation of haustoria, as in *R* gene-mediated resistance HR occurs after the formation of haustoria (Huckelhoven *et al.*, 1999; Trujillo *et al.*, 2004). In Wc-1 the frequency of HR is generally more than the frequency of haustorium formation. Therefore, this nonhost HR could also be a consequence of a flux of non-functional AVR proteins secreted by *Bgt* into the Arabidopsis epidermal cells and mediated through a PAMP-induced pathway (Ellis, 2006).

Similar to *pen1-1* (Collins *et al.*, 2003), the most frequent response to *Bgt* infection in Wc-1 is to surround the infected cell with callose. It was shown that in response to powdery mildew, callose was deposited near the epidermal cell in the apoplast (between the cell wall and plasma membrane) to preclude penetration of the pathogen directly (Bélanger and Bushnell, 2002). Furthermore, there is the assumption that defence substances such as ROIs and phenolics are also accumulated in papillae (Bélanger and Bushnell, 2002).

The additive increased penetration found in F2 individuals of crosses between *eds1* and Wc-1, may indicate that lack of penetration resistance in Wc-1 could possibly operate independently of *EDS1* function. Inhibition of actin microfilament polymerisation in the *eds1* mutant supported increased haustorial formation and enhanced development of secondary hyphae (Yun *et al.*, 2003). In contrast to *eds1,* reduction of actin microfilament polymerisation in Wc-1 did not support further development of secondary hyphae. However inoculation of pre-treated Wc-1 with CE only instigated a 4% increase in haustorial formation. This is an indication of the complexity of NHR in Wc-1. Whereas the weakened basal resistance in *eds1* manifested itself by increased development of *Bgt* as soon as the rearrangement of the cytoskeleton was impaired pharmacologically. The higher number of haustoria due to inhibition of actin microfilament polymerisation was also found in wild type Col-0, which exhibited an early and effective NHR towards *Bgt* (Yun *et al.*, 2003). Manipulation of actin microfilament polymerisation in Wc-1 demonstrates that NHR was only compromised in pre-haustorial resistance and an effective immune system was triggered at the post-haustorial stage. This cell wall weakness was improved upon activation of basal resistance. A similar situation was revealed previously in the lab generated mutant powdery *mildew resistance 4* (*pmr4*). This mutant lacks pathogen-induced callose deposition which is thought to provide a positive barrier for powdery mildew resistance. Interestingly, the mutant exhibited an enhanced resistance rather than the anticipated susceptibility. The loss of callose synthase was overcome with an alternative defence pathway, which was dependent on salicylic acid (Nishimura *et al.*, 2003).

In summary, prehaustorial NHR was effective in the majority of Arabidopsis accessions. Wc-1 plants expressed a significant compatibility towards *Bgt* at this stage as a dilatory response as was obvious due to the moderate fungal growth. During post-haustorial NHR, Wc-1 encased haustoria in callose and the epidermal cell underwent the hypersensitivity response.

Although many questions may be addressed regarding NHR in Wc-1, these can only be answered following cloning and characterisation of the underpinning gene(s).

Chapter 4

Non-Host Disease Resistance in Arabidopsis against the Leaf Rust Pathogen *Puccinia triticina*

Introduction

Non-host resistance (NHR) conveys protection to all varieties of a given plant species against all races of a given pathogen species (Thordal-Christensen, 2003; Heath, 2001; Nurnberger and Lipka, 2005). The presence of this defence system explains why plants are immune to the vast majority of potential pathogens. NHR contrasts with the well studied host resistance, mediated by the products of plant resistance (*R*) genes, which establish pathogen race- or plant cultivar-specific resistance (Dangl and Jones, 2001; Schulze-Lefert and Bieri, 2005). While NHR typically provides durable crop protection in the field, the effectiveness of *R* gene-mediated resistance is routinely transient. Also, while the genetic basis of *R* gene-mediated disease resistance is well defined, the molecular mechanisms underpinning non-host disease resistance remain relatively unexplored.

Pre-formed physical and chemical barriers are thought to constitute the primary tranche of NHR defence mechanisms (Heath, 2000). For example, avenacins, glycosylated plant secondary metoabolites, are required for NHR in oat against the wheat pathogen *Gaemannomyces graminis* var. *tritici* (Papadopoulou *et al.*, 1999). Inducible defence responses, however, are also thought to be important, at least in some cases (Yun *et al.*, 2003; Lipka *et al.*, 2005). These defence mechanisms are likely activated in a non-specific fashion, analogous to the recognition of pathogen associated molecular patterns (PAMPs) in the innate immune response of animals (Akira and Takeda, 2004). In this context, plants are known to perceive a series of PAMP-like molecules including β-glucan and chitin from fungi (Nurnberger *et al.*, 2004) and lipopolysaccharide (Zipfel *et al.*, 2004),

flagellin (Zipfel *et al.*, 2004) and EF-Tu (Zipfel *et al.*, 2006) from bacteria. Furthermore, some of the cognate plant receptors for these molecules have recently been identified: FLS2 and EFR recognize bacterial flagellin (Gomez-Gomez and Boller, 2000) and EF-Tu (Zipfel *et al.*, 2006), respectively.

NHR against biotrophic fungal pathogens that penetrate epidermal cells directly, such as the powdery mildews and the basidospore-derived stage of rust fungi, is routinely expressed as penetration failure (Mellersh *et al.*, 2002). The identification of Arabidopsis mutants that are compromised in penetration resistance expressed against a barley powdery mildew pathogen, *Blumeria graminis* f.sp *hordei* (*Bgh*), has recently led to significant insights into this defence mechanism. Mutations in *PENETRATION* (*PEN*) genes have highlighted possible roles for vesicle trafficking, cell wall remodelling and the extracellular transport of small molecules in NHR against *Bgh* (Collins *et al.*, 2003; Lipka *et al.*, 2005; Stein *et al.*, 2006).

Furthermore, salicylic acid (SA), *ARABIDOPSIS THALIANA S-NITROSOGLUTATHIONE REDUCTASE* (*ATGSNOR*) *1*, *ENHANCED DISEASE SUSCEPTIBILITY* (*EDS*) *1*, *PHYTOALEXIN DEFICIENT* (*PAD*) *4* and *SENESCENCE ASSOCIATED GENE* (*SAG*) *101* also function in a post host cell invasion resistance mechanism against *Blumeria graminis* f.sp *tritici* (*Bgt*) and *Bgh* (Feechan *et al.*, 2005; Lipka *et al.*, 2005; Yun *et al.*, 2003). Concurrent impairment of both pre- and post plant cell invasion defence mechanisms converts Arabidopsis into a host for these biotrophic pathogens (Lipka *et al.*, 2005; Yun *et al.*, 2003).

While the mechanisms of NHR against powdery mildew fungi are beginning to be uncovered, the nature of this defensive bulwark against other fungal pathogens with alternative lifestyles remains largely unexplored. *Puccinia triticina* (*Ptr*) is an obligate biotrophic pathogen of wheat that is of major economic significance. This pathogen is known to establish a complex and intimate association with its host (Mendgen *et al.*, 1996). For this part of my reserach the purpose was to

investigate the *Ptr*-Arabidopsis pathosystem, which we have utilized to explore the expression of NHR against this fungus. In this context, I analysed the defence responses of Arabidopsis and the natural variation among Arabidopsis accessions. I also performed a QTL analysis to study the genetic basis of this nonhost interaction. The restriction of *Ptr* growth was predominantly expressed before host cell invasion. Inoculation of this fungus onto Arabidopsis triggered the rapid induction of a variety of defence responses. Analysis of natural variation revealed that the Wa-1 accession exhibited significantly increased susceptibility to *Ptr*. Furthermore, this Arabidopsis line showed strikingly enhanced accumulation of camalexin, elevated levels of SA and accelerated kinetics of SA-dependent gene expression. Using a population of recombinant inbred lines (RILs) a number of quantitative trait loci (QTLs) were identified that underpinned natural variation in Arabidopsis against *Ptr*.

Results

Ptr urediospores alighting onto the surface of a wheat leaf in the presence of water or in high relative humidity germinate to produce germ tubes, which utilize topographical and chemical cues to locate stomata (Collins *et al.*, 2001). The fungus then undergoes a defined series of developmental stages (Fig. 4.1A) (Mendgen *et al.*, 1996). First, an appressorium is generated which produces a penetration peg that enters the stomatal opening. The fungus then develops a sub-stomatal vesicle, infection hyphae and subsequently a haustorial mother cell, which forms adjacent to a host mesophyll cell. Following penetration of the plant cell wall a haustorium then forms in an invagination of the host plasma membrane. This structure withdraws nutrients from the invaded plant cell that subsequently supports the development of a network of intercellular hyphae and further intracellular haustorium formation. Within 8 to 10 days following infection a uredium is produced which releases additional urediospores following

the collapse of the epidermal cell layer, potentially spreading the infection to other plants.

Growth and Development of *Ptr* on Arabidopsis

Urediospores from the *Ptr* isolate WBRS-97-3 (Zhang *et al.*, 2003) were applied onto the leaves of the Arabidopsis accession Col, a non-host plant for this pathogen. Individual urediospores produced a germ tube in 91±2.2% of cases, which grew randomly over the Arabidopsis leaf surface (Fig. 4.1B). In a similar fashion, 95±2.3% and 93±2.3% of urediospores produced germ tubes on a susceptible and resistant wheat cultivar, respectively. Only 12±3.6% of urediospores, however, produced a germ tube that successfully located a stoma and formed an appressorium on Arabidopsis (Fig. 4.1C). In contrast 85±2.3% and 83±2.6% of *Ptr* germ tubes located a stoma on susceptible and resistant wheat cultivars, respectively. Interestingly, many stoma penetration attempts in Arabidopsis resulted in the hypersensitive response-like cell death of the cognate guard cells (Fig. 4.1D). This guard cell death (GCD) suppressed further *Ptr* growth. Subsequent to successful stoma penetration, *Ptr* produced a sub-stomatal vesicle and infection hyphae (Fig. 4.1E), followed by a haustorial feeding structure (Fig. 4.1F), within a penetrated mesophyll cell. It is noteworthy that *Ptr* haustorium developed at a very low frequency: only 0.2±.01% of the inoculated urediospores on Arabidopsis produced a haustorium. On susceptible (Fig. 4.1G) and resistant wheat cultivars 72±4.1% and 4±2.7% respectively of urediospores went on to develop haustoria. In Arabidopsis many of these haustoria became encased in deposits containing callose (Fig. 4.1H), in a similar fashion to that which occurs during an incompatible interaction in wheat (Anguelova *et al.*, 1999).

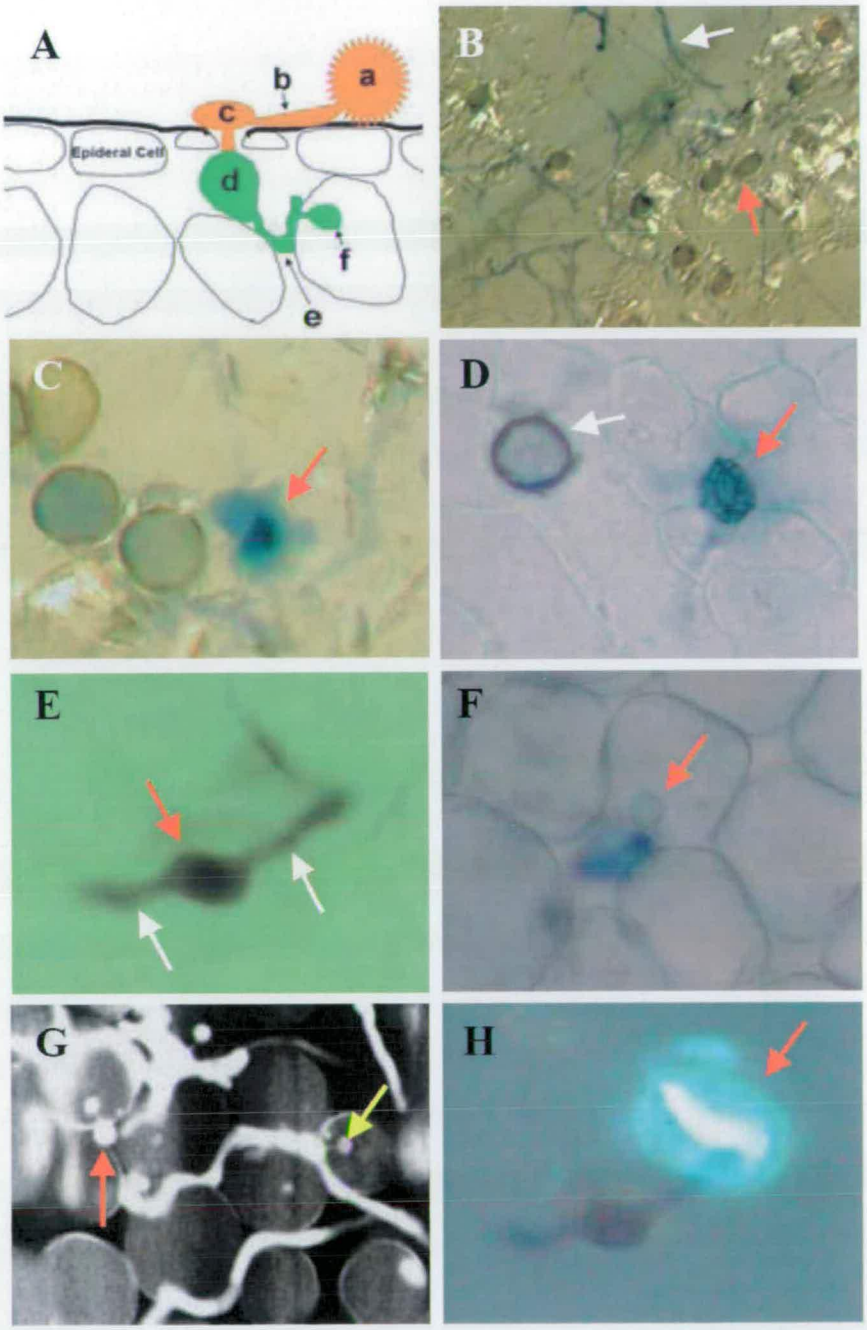


Figure 4.1. Plant responses to attempted *Ptr* infection. A) Schematic depicting *Ptr* infection of wheat. a, indicates *Ptr* urediospore on the surface of a wheat leaf; b, shows a *Ptr* germ tube; c, marks a *Ptr* appressorium on the surface of a stoma; d, indicates a sub-stomatal vesicle; e, shows an infection hypae; and, f, a *Ptr* haustorium within a penetrated host mesophyll cell; B) Shows germinated *Ptr* urediospores. The red arrow marks an urediospore, a white arrow indicates a *Ptr*

(Fig 4.1. countinued) germ tube on the Arabidopsis leaf surface; C, A) *Ptr* appressorium formed over a stoma, marked by the red arrow; D) Guard cell death (GCD), indicated by a red arrow, following attempted stoma penetration by *Ptr*. The white arrow points to an adjacent *Ptr* urediospore, with cognate germ tube out of the focal plane; E) The red arrow shows a sub-stomatal vesicle formed within the sub-stomatal cavity. The white arrows point to infection hyphae within the sub-stomatal cavity; F) The red arrow indicates a *Ptr* haustorium which has developed within a penetrated Arabidopsis mesophyll cell; G) A haustorial mother cell is shown by the red arrow. The yellow arrow points to a haustorium that has developed within a wheat mesophyll cell; H) A haustorium formed within a penetrated Arabidopsis mesophyll cell has become encased in a deposit of callose, shown by the red arrow.

Establishment of NHR against *Ptr* Correlates with the Activation of Defence Responses

Attempted *Ptr* infection of Arabidopsis Col plants resulted in the rapid production of reactive oxygen intermediates (ROIs) (Grant *et al.*, 2000; Lamb and Dixon, 1997) at approximately 24 hours post inoculation (hpi), as detected by 3-3'-diaminobenzidine (DAB) staining (Fig. 4.2A) (ThordalChristensen *et al.*, 1997). This response was absent during a compatible interaction of *Ptr* with its host plant, wheat (Fig. 4.2A). In Arabidopsis, ROI synthesis is thought to be mediated by homologs of the mammalian gp^{91phox} respiratory burst NADPH-oxidase sub-unit encoded by a ten-member gene family (*AtRBOHA-AtRBOHI*) (Torres *et al.*, 1998; Keller *et al.*, 1998). Mutations in either *AtRBOHD* or *AtRBOHF* reduce ROI generation in response to *Pseudomonas syringae* pv. *tomato* expressing the avirulence gene *avrRpm1* or the Emoco5 isolate of *Hyaloperonospora parasitica* (Torres, 2002). In response to *Ptr* challenge, there was a significant reduction in ROI generation in *atrbohD* plants and no significant increase in these intermediates in the *atrbohF* line (Fig. 4.2B). Furthermore, ROI production was also not significantly induced in *atrbohD atrbohF* plants (Fig. 4.2B-G). Despite the reduction in *Ptr* triggered ROI levels, these lines did not support increased *Ptr* stoma penetration, sub-stomatal vesicle formation or haustorium development relative to wild-type plants (Fig. 4.3A).

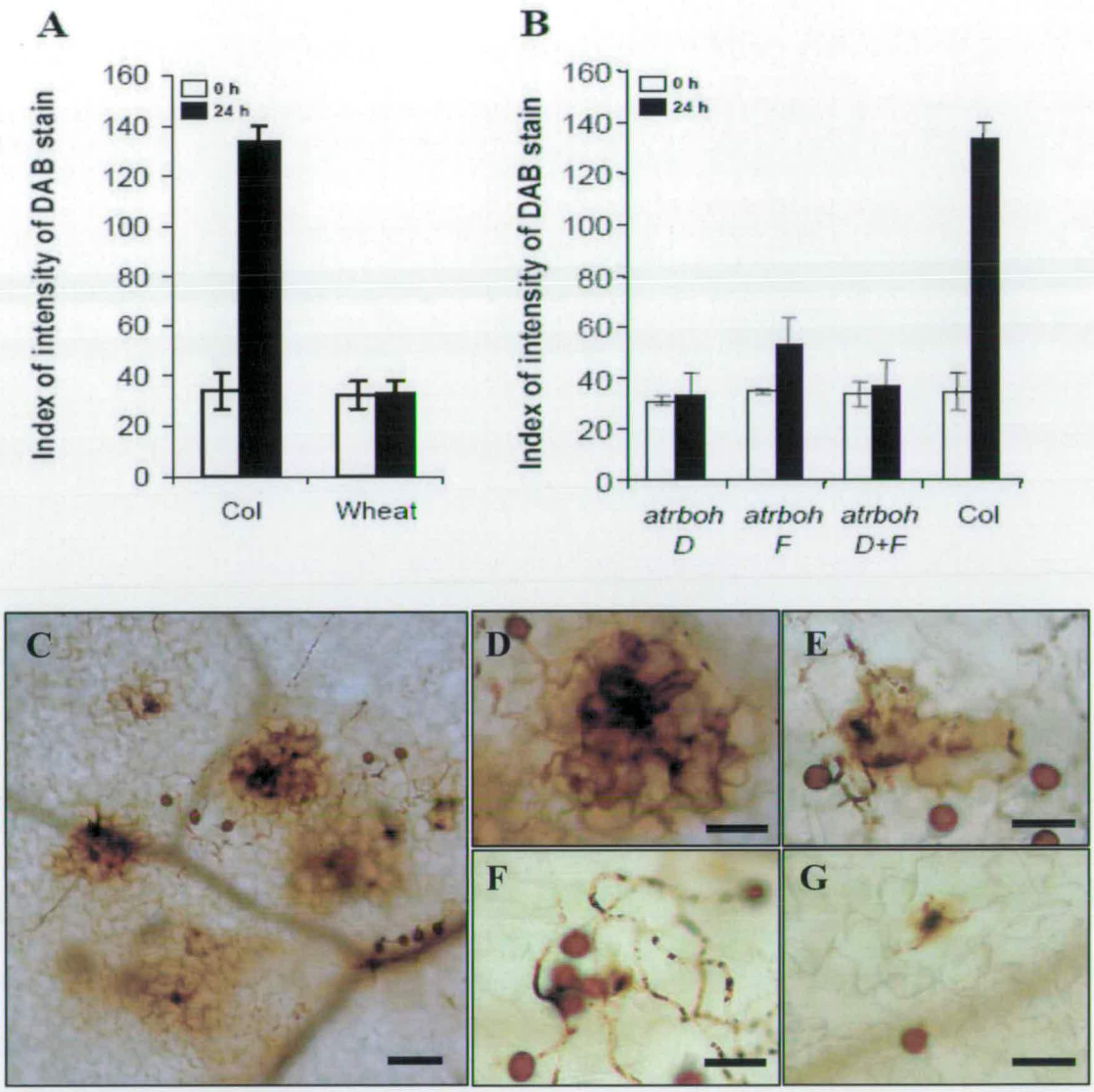
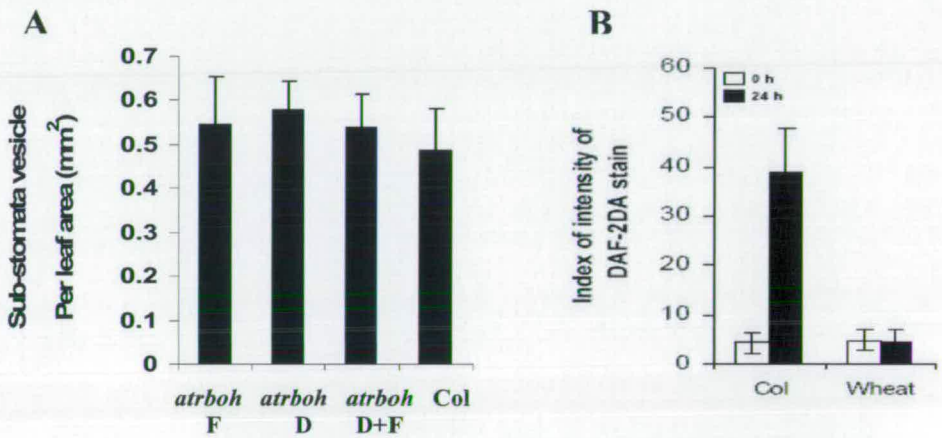


Figure 4.2. A) Attempted *Ptr* infection of Arabidopsis Col plants induces the accumulation of ROIs as determined by the quantification of DAB staining. In contrast, a compatible interaction of *Ptr* with its host plant, wheat, fails to invoke significant generation of ROIs. B, ROI accumulation in response to *Ptr* challenge is reduced in Arabidopsis *atrboh* mutants; C,D,E,F,G) Reactive Oxygen Intermediate (ROI) accumulation in Arabidopsis Col(C) lower magnification; Col(D); *atrboh* F (E); *atrboh* D (F); *atrboh* D+F (G) around stomata region , 24 hours after challenge. Leaves were inoculated with *Puccinia triticina*. In C,D,E,F,G conidium (arrowhead) and hydrogen peroxide (arrow) shown. Bars in D,E,F,G are 60μm and in C is 150μm. Error bars represent SE. These experiments were repeated three times with similar results.

Attempted *Ptr* infection of Arabidopsis also triggered the accumulation of nitric oxide (NO) (Wang *et al.*, 2006) at 24 hpi, as scored by quantified 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescence (Zeidler *et al.*, 2004), detected by confocal imaging (Fig. 4.3B). There was a striking decrease in DAF-2DA staining following application of the NO scavengers CPTIO and rutin trihydrate, confirming the specificity of this procedure (Correa-Aragunde *et al.*, 2006). We did not observe significant NO production during the compatible interaction of *Ptr* with wheat (Fig. 4.3B-F).



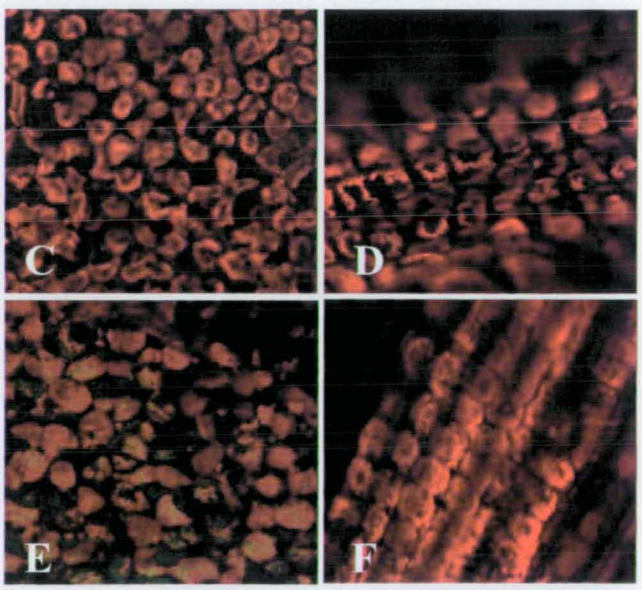


Figure 4.3. A) Despite of decreased amount of ROI in *atrboh* mutants, the number of sub-stomatal vesicle formed per square millimetre of leaf surface remained unchanged; B) Attempted *Ptr* infection of Arabidopsis Col plants induced the accumulation of NO as determined by the quantification of DAF-2DA staining. C-D) Col and wheat at 0 hpi; E-F) Col and wheat at 24 hpi, NO stained as green blots on Col. Error bars represent SE. These experiments were repeated three times with similar results.

Attempted *Ptr* infection also induced the transient expression of a luciferase (*LUC*) reporter gene cued by ROIs (Grant *et al.*, 2000), SA (Grant *et al.*, 2003) or jasmonic acid (JA) (Love *et al.*, 2005; Yun *et al.*, 2003) (Figure 4.4A-C).

The accumulation of SA is sometimes a key feature underpinning the establishment of NHR (Feechan *et al.*, 2005). Thus, we determined the concentration of total SA, consisting of both free SA and SA-β-glucoside (SAG), in *Ptr* challenged Col plants. The concentration of total SA significantly increased in response to attempted *Ptr* infection (Fig 4.5A). We therefore investigated the individual levels of both free SA and SAG. The levels of both free SA and SAG were elevated: free SA levels increased by 169% and SAG levels by 651%, relative to *Ptr* challenged Col plants (Fig 4.5B and C).

We also measured the concentration of camalexin, an indole derived phytoalexin found in Arabidopsis, which is required for effective disease resistance against some fungal pathogens (Zhou *et al.*, 1999). There was a small but significant elevation in camalexin levels following *Ptr* challenge (Fig. 4.5D). Thus, in Col plants at 48 hpi of *Ptr* there was 137% increase in the concentration of this metabolite compared to the concentration found in unchallenged control plants.

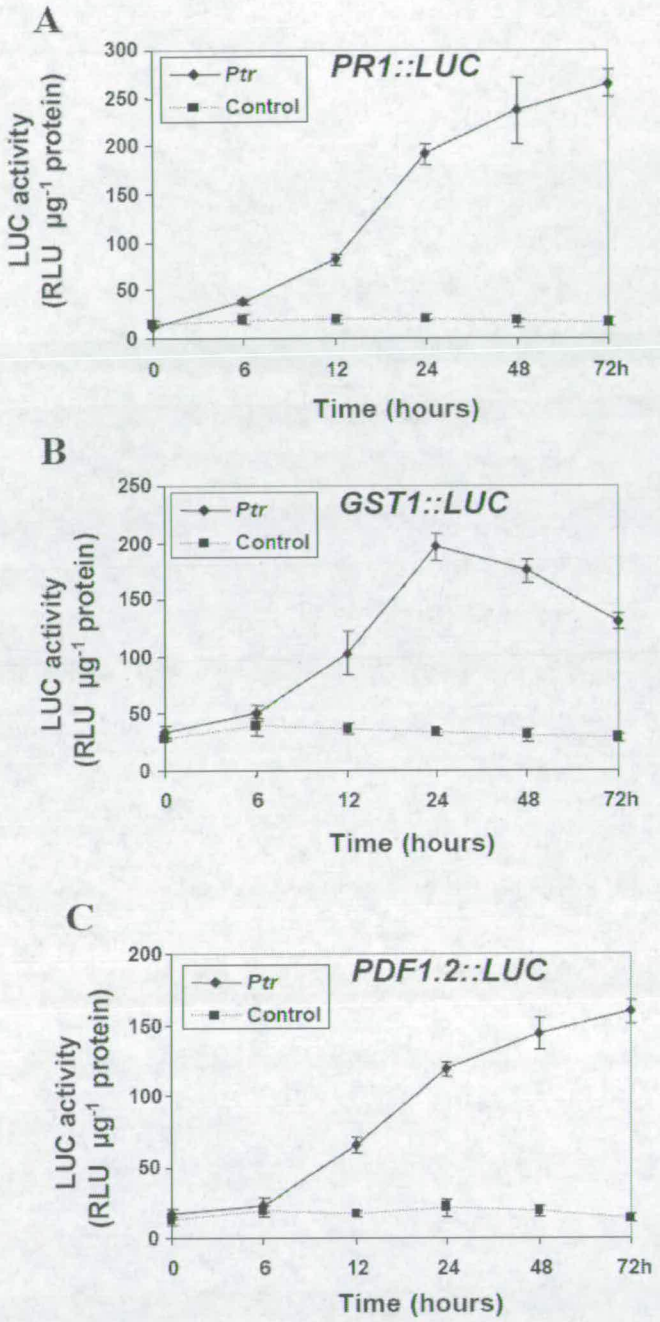


Figure 4.4. Profiles of defence gene expression established by LUC imaging following *Ptr* challenge. A) *PR1::LUC* gene expression; B) *GST1::LUC* induction; C) *PDF1.2::LUC* gene expression. Each transgenic line was either challenged with *Ptr* (filled diamonds) or mock inoculated (filled squares) as a control and LUC activity was determined over time as indicated. Error bars represent 95% confidence intervals. These experiments were repeated three times with similar results.

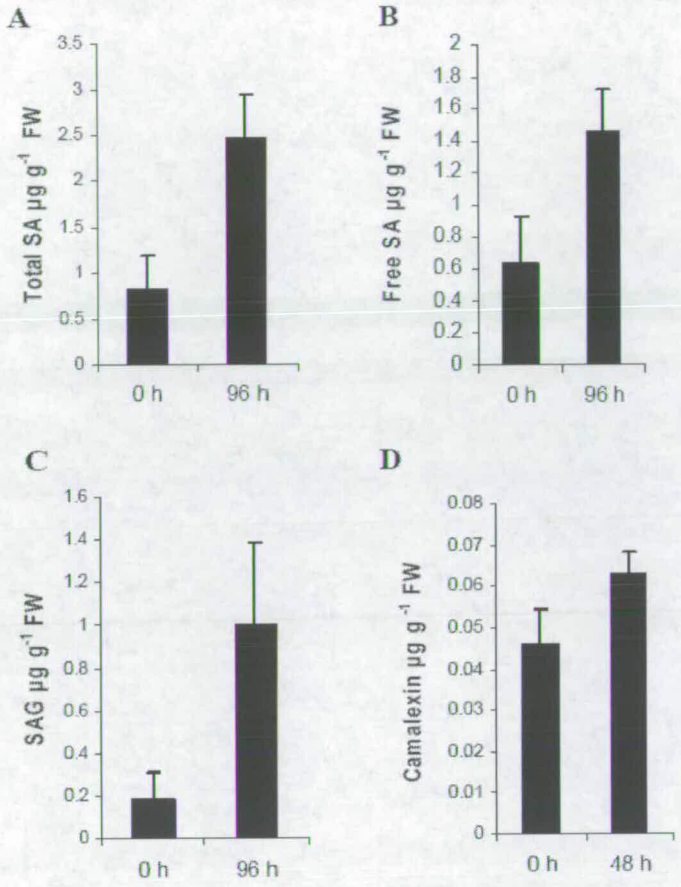


Figure 4.5. *Ptr* challenge induces the accumulation of SA and camalexin in Arabidopsis. A) Concentration of total SA at 0 and 96 hpi of *Ptr*; B) Determination of free SA levels following *Ptr* inoculation at the times indicated; C) Induction of SAG accumulation following attempted *Ptr* infection of Arabidopsis; D) Accumulation of camalexin in Arabidopsis at 0 and 48 hpi of *Ptr*. Error bars represent 95% confidence intervals. These experiments were repeated twice with similar results.

Response of Arabidopsis Defence-Related Mutants to *Ptr* Infection

To investigate if any defined defence signalling pathways were required to establish NHR against *Ptr* we challenged a series of key Arabidopsis defence-related mutants. Thus, *Ptr* was inoculated onto *coronatine insensitive* (*coi*) *1* (Feys *et al.*, 1994), *ethylene insensitive* (*ein*) *2* (Bent *et al.*, 1992), *SA-induction*

deficient (*sid*) *1* and *2* (Nawrath and Metraux, 1999), *non-expresser of PR genes* (*npr*) *1* (Cao *et al.*, 1997) mutants and SA-depleted (*nahG*) (Delaney *et al.*, 1994) plants. We also tested *phytoalexin deficient* (*pad*) *3* and *4* (Glazebrook and Ausubel, 1994; Zhou *et al.*, 1999), *suppressor of G2 allele of skp1-4* (*sgt*) *1* (Austin *et al.*, 2002), *pen1* (Collins et al., 2003), *pen2* (Lipka *et al.*, 2005), *pen3* (Stein *et al.*, 2006) and *required for MLA resistance* (*rar*) *1* (Muskett *et al.*, 2002; Tornero *et al.*, 2002) mutants. None of these Arabidopsis lines exhibited a significant increase in *Ptr* sub-stomatal vesicle frequency (SVF). Indicating that these pathways alone do not play crucial role in this pathosystem.

Natural Variation of NHR in Arabidopsis against *Ptr*

To explore potential variation in the expression of NHR in Arabidopsis, we challenged 79 geographically diverse accessions of this plant with an equal amount of *Ptr* urediospores and scored SVF in these accessions. The name and origin of these accessions is listed in Material and Methods. Seven days post infection (dpi) of the plants, marked leaves were detached and stained with trypan blue. Trypan blue staining facilitates the visualization of dead cells and fungal structures. Due to NHR, most appressoria failed to penetrate through stomata on the vast majority of accessions. However, the formation of appressorium germ tube (AGT) was highly frequent.

We scored number of "Guard Cell Death (GCD)" and "Sub-stomatal Vesicles formed (SVF)" per square millimetre of the leaf surface of the Arabidopsis seedlings. The experiment carried out in a Randomised Complete Block (RCB) design with three replications to minimise the effect of environmental factors on *Ptr*-Arabidopsis interaction. Data for both traits were transformed for normalisation purposes (Miller, 1997). An analysis of variance (ANOVA) did not detect any significant differences between replications for both GCD and SVF. Despite the significant variation for GCD and SVF observed among accessions,

except Wa-1, the variation was not considerable and thus not subjected to further analysis.

In the Col reference line the number of SVF was 0.49 per mm^2 of Arabidopsis leaf surface. The other accessions examined exhibited a similar level of SVF to that of the Col accession, with a range between 0.48 and 0.93 per mm^2. The results for a subset of these accessions are shown in Fig. 6.4A. In contrast, the Wa-1 accession showed a striking increase in SVF following *Ptr* challenge: 2.04 per mm^2 of Arabidopsis leaf surface (Fig. 4.6A). Moreover, the Pi-0 line, which supported a SVF of 0.19 per mm^2, exhibited a marked reduction in SVF relative to the Col reference line.

To study the existence of any possible relationship between these GCD and SVF, Pearson correlation analysis was performed on the least-square means averages for each pair of characteristics pair. Correlation analysis showed a significant negative correlation between the accessions for these two traits ($r = -0.34$, $\alpha < 0.01$) suggesting there is an antagonistic interaction between SVF and GCD. This suggests that GCD inhibits further *Ptr* penetration and development. Likewise, in the absence of GCD occurrence, the pathogen has better chances of developments.

As the Wa-1 line supported enhanced *Ptr* SVF, we investigated the expression of defence responses in this accession. First, we determined the concentration of camalexin at 48 hpi of *Ptr*. Wa-1 plants showed a striking increase in the concentration of this key defence metabolite. Thus, *Ptr* challenged Wa-1 plants accumulated 4037% more camalexin than the Col reference line (Fig. 4.6B).

The Wa-1 line, in response to attempted *Ptr* infection, also accumulated increased total SA, free SA and SAG compared to Col plants (Fig. 4.6C-E): free SA levels were elevated by 169% and SAG levels by 651% relative to *Ptr* challenged Col plants. However, it should be noted that Wa-1 plants also possessed a higher basal level of SAG.

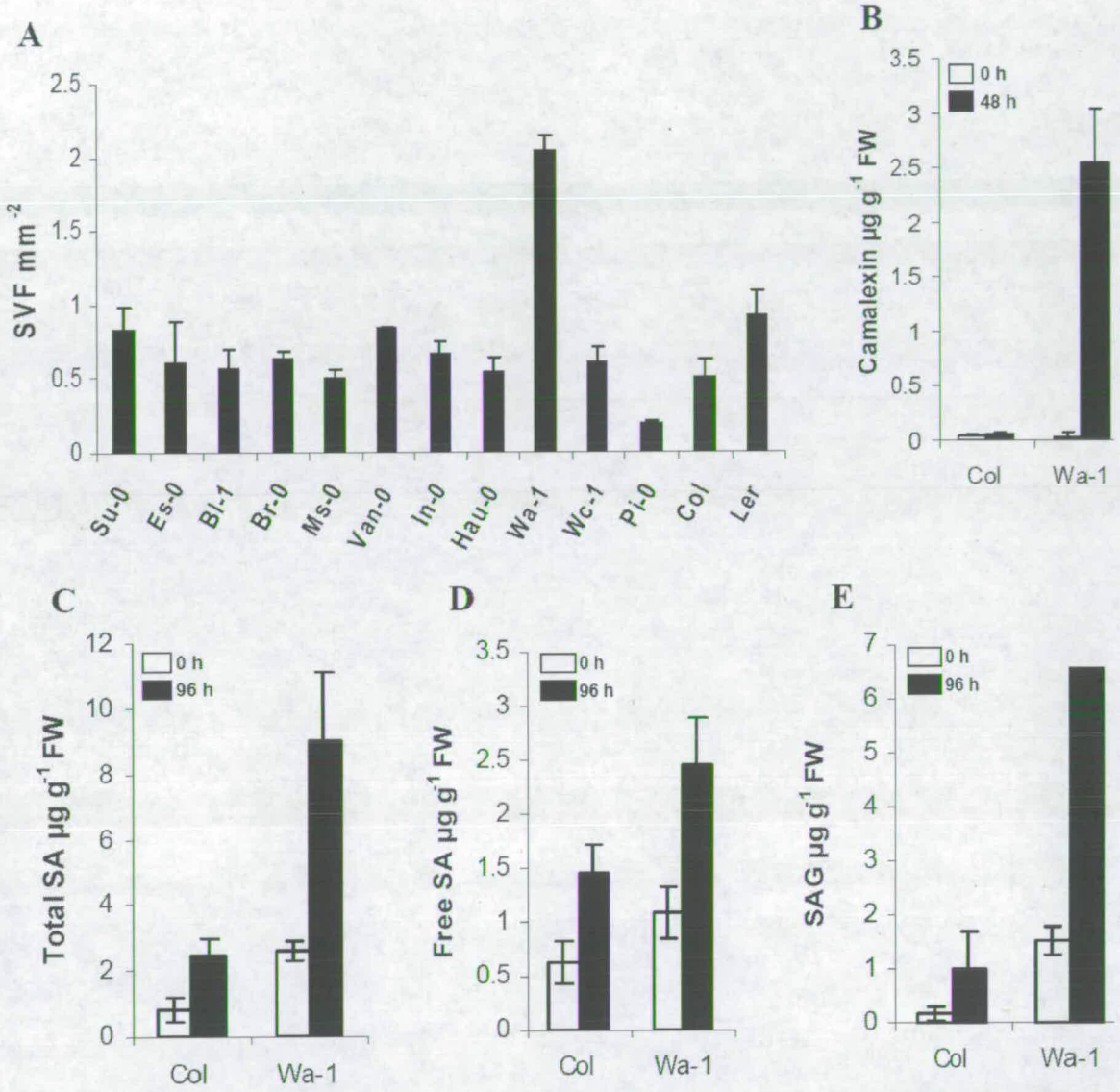


Figure 4.6. Natural variation among Arabidopsis accessions in their response to *Ptr*. A) Natural variation in a variety of geographically diverse Arabidopsis accession with respect to the level of SVF following attempted *Ptr* infectio; B) Induction of camalexin accumulation in Col and Wa-1 lines at 48 hpi of *Ptr*; C – E) Measurement of total SA (C), free SA (D) and SAG levels (E) in response to attempted *Ptr* infection. Error bars represent 95% confidence intervals. All these experiments were repeated at least twice with similar results.

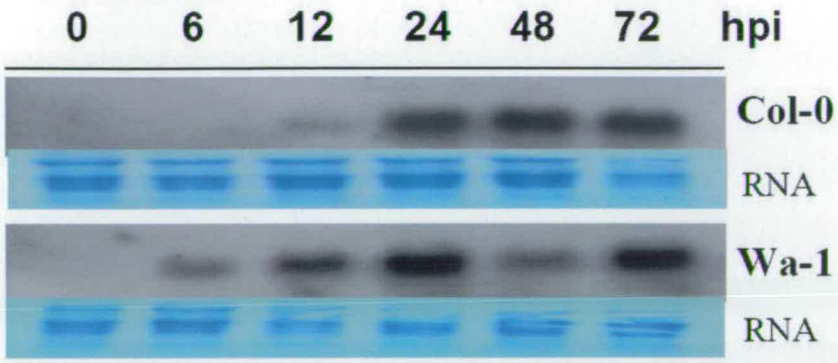
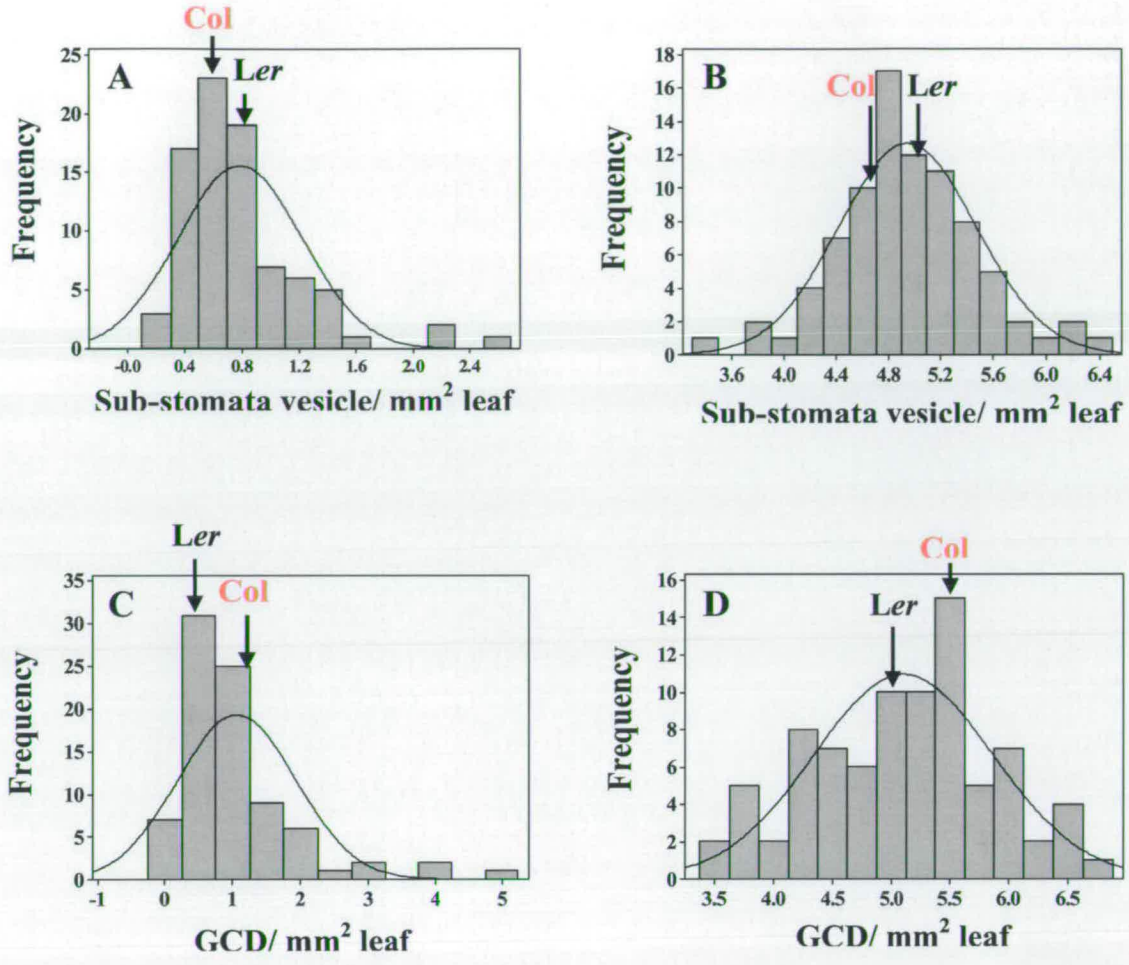


Figure 4.7. Northern analysis of *PR1* gene expression in Col and Wa-1 accessions following *Ptr* inoculation.

We also examined SA signalling in Wa-1 plants following attempted *Ptr* infection, by scoring the expression of the SA marker gene, *PR1* (Uknes *et al.*, 1992). The expression of this gene was first detected at 24 hpi of *Ptr* in Col plants (Fig. 4.7). In contrast, *PR1* transcripts were first observed at 6 hpi following *Ptr* challenge in Wa-1 plants (Fig. 4.7).

Identification of QTLs for NHR in Arabidopsis against *Ptr*

While this research was running, it was revealed that Wa-1 is a natural autotetraploid Arabidopsis accession (Henry *et al.*, 2005). However, the triploid F1 offspring of the cross between Col and Wa-1 is fertile and their F2 population is a pack of different aneuploids. Development of this F2 population for a few generations resulted in diploid and tetraploid cohorts (Henry *et al.*, 2005). Consequently, the Recombinant Inbred Lines (RIL) produce from Col and Wa-1 (Schiff *et al.*, 2001) was collected from Arabidopsis stock centre.



E

S.O.V	df	SVF (SS)	GCD (SS)
Genotypes	**101**	**56.31 ***	**96.750 *****
Replication	**1**	**1.008 ns**	**0.068 ns**
Error	**101**	**33.225**	**27.800**
R^2		**57.42**	**77.75**

Figure 4.8. Distribution of RILs for Guard cell death (GCD) and Sub-stomatal vesicle frequency (SVF) along with the position of their parental lines, Col and L*er*. A,C) raw data which do not fit with normal distribution; B,D) transformed data which fits with normal distribution; E) results of analysis of variance shows significant variation for GCD and SVF among RILs. R^2, indicates the percentage of variation among RILs due to the corresponding characteristic. SOV, source of variation; df, degree of freedom; SS, sum of square.

Therefore, in order to examine the genetic basis underlying the natural variation in the responses of Arabidopsis to *Ptr,* we utilized a well established set of RILs, derived from a Col x L*er* cross (Lister and Dean, 1993). There are higher chances of detecting small effect of gene(s) in this set of RIL as they are very well genotyped. This resource facilitates the rapid evaluation of genetic variation and the ready ability to map any quantitative trait loci (QTL) controlling this variability (Maloof, 2003). We exploited this approach to explore the genetic basis underlying *Ptr* triggered GCD and SVF. In this context, we scored a population of 102 RILs for either GCD or SVF following *Ptr* inoculation. Although Col and L*er* exhibited relatively small differences between these traits, transgressive variation in both directions were observed in the RIL population, indicating the presence in the two parental lines of alleles increasing and reducing the extent of GCD and SVF (Fig. 4.8A-D). Thus, the RIL population showed highly significant differences for these traits (Fig. 4.8E). The heritabilities for GCD and SVF were 0.691 and 0.464 respectively.

There are small but continuous variation for traits and the differences between segregating offspring are significant as well. Therefore in these situations, mapping a characteristic is not as simple as a single gene controlling a qualitative trait. These findings prompted us to search for possible QTLs associated with these traits. This was undertaken by employing composite interval mapping (CIM) (Zeng, 1993) and multiple interval mapping (MIM) (Kao *et al.*, 1999) with QTL Cartographer software (Wang *et al.*, 2005). Composite interval mapping adds background loci to simple interval mapping and reduces the number of "ghost" QTL (Zeng, 1993). MIM method uses multiple marker intervals simultaneously to fit multiple putative QTL directly for mapping QTL. The precision and power of QTL mapping has been improved in MIM, as this method is able to estimate and analyze epistasis between QTL, genotypic values of individuals, and heritabilities of quantitative traits (Kao *et al.*, 1999).

CIM analysis identified two QTLs associated with GCD, the most significant of which appeared to be located on chromosome 1, positioned from 49.91 – 55.81

cM (Table 4.1). This QTL had a Logarithm of Odds (LOD) score of 3.78, which accounted for 12.53% of the total variation for this trait. Collectively, these two QTLs for GCD accounted for 21.34% of the total variation. CIM analysis established that the extent of SVF was governed by three QTLs, on chromosomes 2, 4 and 5 (Table 4.1). LOD scores for these QTLs were 4.56, 3.52 and 4.21, respectively. Together these QTLs represented 40.59% of the total variation for this trait. Our analysis also did not detect any epistasis between these QTLs. Furthermore, QTLs identified using the CIM method (Fig 4.9) were similar to those uncovered with MIM analysis, except the QTL on chromosome 3 for GCD, which was not found to be significant through the MIM method.

Table 4.1. Arabidopsis QTL governing *Ptr* triggered GCD and the extent of *Ptr* SVF determined by CIM analysis.

Characteristics		Chromosome	Position (cM)	LOD	% of Variation explained	Additive effect
Sub-Stomata Vesicle Frequency (SVF)	QTL 1	2	90.49	4.56	16.47	-2.37*
	QTL 2	4	25.09	3.52	11.85	-1.99
	QTL 3	5	119.04	4.21	12.27	1.94
Guard Cell Death (GCD)	QTL 1	1	49.91	3.78	12.53	2.84
	QTL 2	3	56.3	2.54	8.81	2.31

* A negative or positive sign for additive effect indicates the given QTL was derived from either the L*er* or Col accession, respectively.

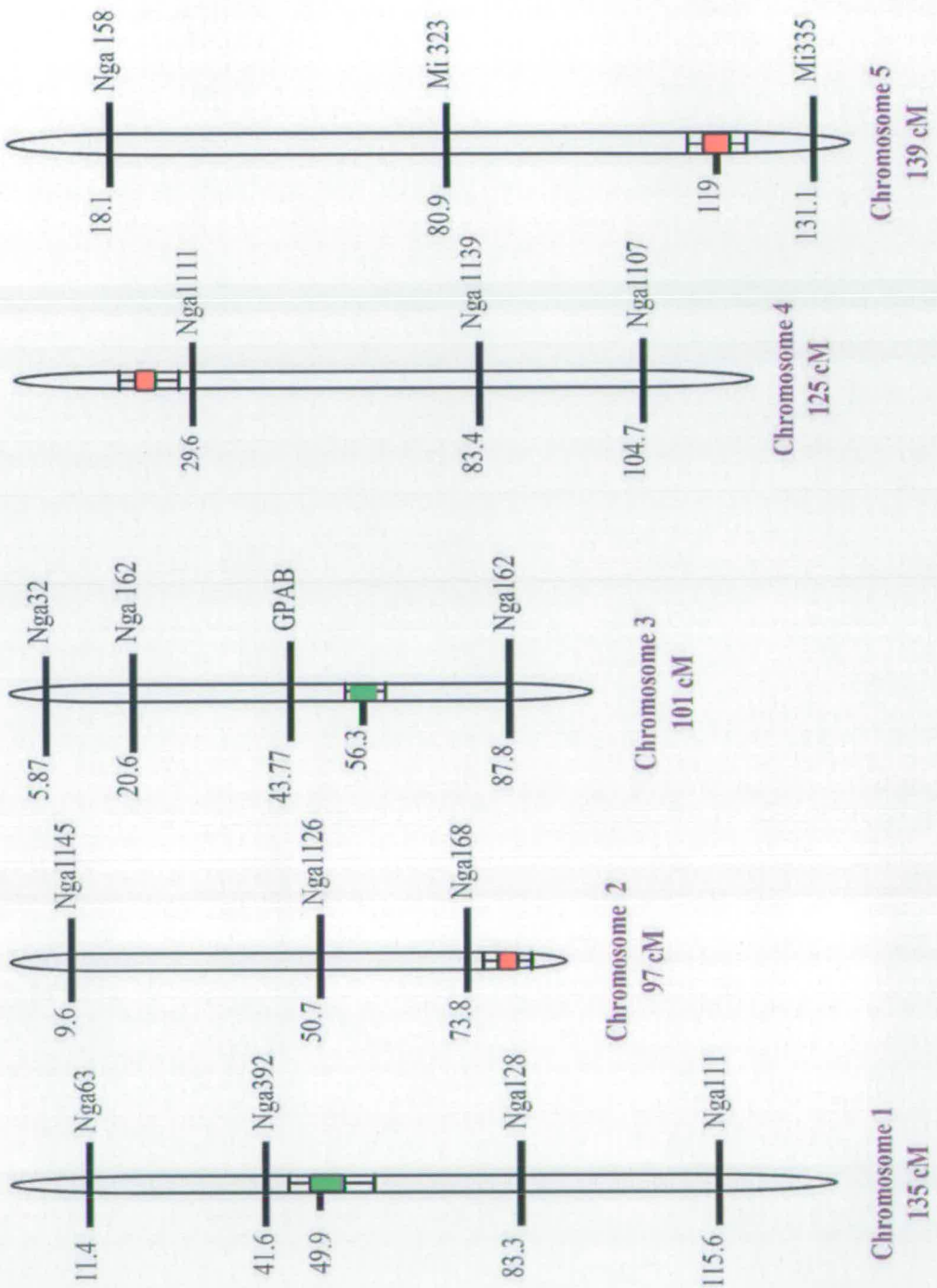


Figure 4.9. Linkage map of five chromosomes of Arabidopsis and the QTLs identified for "Guard cell death (GCD)" and "sub-stomata vesicle (SVF)". Red coloured regions are the QTL detected for SVF and the Green regions are QTL for GCD. Error bars for QTLs cover 95% confidence intervals.

Discussion

While the vast majority of *Ptr* infection attempts failed before host mesophyll cell penetration, attempted *Ptr* infections nevertheless induced a number of plant defence mechanisms. One of the most rapid of these was the production of ROIs and NO. Mutations in *AtRBOHD* significantly reduced ROI production while loss of *AtRBOHF* function resulted in no significant ROI accumulation following inoculation of *Ptr*. These findings suggest a role for both of these two related proteins in *Ptr*-triggered ROI generation; however, AtRBOHF appeared to make the greatest contribution. Conversely, in response to avirulent *Pst*DC3000 strains and *H. parasitica* isolates, AtRBOHD was responsible for the majority of the ROI generated (Torres *et al.*, 2002). The relative contribution of individual AtRBOH proteins may therefore be dependent upon the identity of the pathogen that triggered a given oxidative burst. Furthermore, the absence of significant ROI accumulation in either the *atrbohF* or *atrbohD atrbohF* lines did not lead to an increase in SVF, suggesting this defence mechanism is either dispensable or functionally redundant with respect to resistance preceding host cell invasion.

The utilization of reporter constructs and their cognate endogenous marker genes, established that attempted *Ptr* infection induced SA, JA and ROI signalling. In contrast, the expression of NHR against *Bgt* did not include JA signalling (Yun *et al.*, 2003). Therefore, highlighting a potential mechanistic difference between the deployment of defence responses in Arabidopsis against these two non-adapted fungal pathogens. Furthermore, the induction of SA- and ROI-dependent genes was significantly stronger following *Bgt* challenge (Yun *et al.*, 2003) compared to attempted *Ptr* infection. Presumably, this reflects the increased level of plant cell death following attempted *Bgt* infection compared to the relatively low level of host cell death observed following *Ptr* challenge. The magnitude of SA-dependent gene expression is thought to correlate with the extent of pathogen triggered host cell death (Ferrari *et al.*, 2003). Interestingly, *Ptr* challenge induced the accumulation of the Arabidopsis phytoalexin, camalexin (Zhou *et al.*, 1999). Mutations which compromise camalexin synthesis have been shown to

compromise basal resistance against the fungal pathogen *Alternaria brassicicola* (Thomma *et al.*, 1999) but not *Pseudomonas syringae* pv. *maculicola* ES4326 (Zhou *et al.*, 1999). The Arabidopsis *pad3* mutant line (Zhou *et al.*, 1999), which exhibits reduced camalexin synthesis, did not support increased growth of *Ptr*, suggesting this phytoalexin does not make a major contribution to the expression of NHR against this particular fungal pathogen or alternatively its role is functionally redundant.

Analysis of a series of well characterized defence-response mutants following *Ptr* challenge revealed that none of these lines were significantly more susceptible to *Ptr*. Thus, neither the formation of pre-host cell invasion structures such as sub-stomatal vesicles or post-host cell invasion structures such as haustoria, were increased in any of these mutant Arabidopsis lines. These data contrast with that of attempted infection of Arabidopsis by *Bgt* or *Bgh* where *AtGSNOR*, *EDS1*, *PAD4* and *SAG101*, in addition to SA accumulation, are required for the deployment of NHR following host cell invasion (Yun *et al.*, 2003; Lipka *et al.*, 2005; Feechan *et al.*, 2005). In a similar fashion, full expression of post host cell invasion defences against the cowpea rust fungus *Uromyces vignae*, also requires the accumulation of SA in Arabidopsis (Mellersh and Heath, 2003). Collectively, these observations highlight potential differences in the emphasis of NHR mechanisms in Arabidopsis against *Ptr* compared to either *U. vignae* or non-adapted powdery mildews. Thus, while the number of haustoria formed per fungal spore on wild-type Arabidopsis plants is 30% and 5% for *U. vignae* or *Bgh* respectively (Lipka *et al.*, 2005; Mellersh and Heath, 2003), only 0.2% of *Ptr* infection attempts resulted in the development of a haustorium. Therefore, NHR mechanisms that precede host cell invasion appear to undertake the predominant role in the restriction of attempted *Ptr* colonization. In contrast, defence responses in Arabidopsis deployed post host cell invasion play an important function in the constraint of infection by either *U. vignae* or non-adapted powdery mildews (Lipka *et al.*, 2005; Mellersh and Heath, 2003; Yun *et al.*, 2003).

The exploitation of natural variation in Arabidopsis is a powerful tool for gene discovery (Borevitz and Chory, 2004). In this context, we assessed the level of NHR against *Ptr* established by 79 geographically diverse Arabidopsis accessions. The Wa-1 accession exhibited a striking increase in the formation of *Ptr* sub-stomatal vesicles in comparison to the other lines tested. Therefore, either the Wa-1 line may exhibit reduced resistance to *Ptr* preceding host cell invasion or show enhanced expression of susceptibility factors towards this pathogen. Pre-formed chemical barriers are known to function as important determinants of NHR. Thus, a mutant oat line deficient in the production of avenacins was compromised in the expression of NHR against the wheat pathogen *Gaeumannomyces graminis* var *tritici* (Papadopoulou *et al.*, 1999). In a similar fashion, the decreased synthesis or secretion of one or more extracellular chemicals may therefore reduce resistance against *Ptr* in Arabidopsis preceding mesophyll cell invasion. With respect to enhanced expression of susceptibility factors, the interpretation of both topographical signals and chemical cues, such as hexonols, by *Ptr* may be important for the location of stomata in its natural host, wheat (Collins *et al.*, 2001; Grambow and Riedel, 1977). Thus, variations in chemical signals or leaf architecture in Wa-1 plants may improve the ability of *Ptr* to recognize stomata and develop appropriate appressoria, leading to increased susceptibility towards this pathogen in Wa-1 plants.

While Wa-1 plants exhibited enhanced susceptibility to *Ptr*, as determined by increased SVF, counter-intuitively key defence responses in this accession were both stronger and more rapid compared to Col reference plants and all other tested accessions. Thus, camalexin accumulation in Wa-1 plants was significantly greater in response to attempted *Ptr* infection. Furthermore, Wa-1 plants also exhibited increased accumulation of SA and SAG and expedited kinetics of *PR1* gene expression following *Ptr* challenge. Collectively, these data support the notion that the enhanced growth of *Ptr* in the sub-stomatal cavity in Wa-1 plants triggered more powerful defence responses in this line. The Wa-1 line therefore constitutes an important reservoir of natural variation in the expression of NHR against *Ptr* that precedes host cell invasion.

As a well characterized population of RILs from a Col x L*er* cross was readily available (Lister and Dean, 1993), we exploited this collection to explore the genetic control of NHR against *Ptr*. This analysis defined five QTL that govern NHR against *Ptr*. Between individuals there was significant variation in the extent of both GCD and SVF. Using CIM and MIM in conjunction with QTL Cartographer software, we identified two QTLs governing GCD, the most significant of which appeared to be located on chromosome 1. Furthermore, three unrelated QTLs were uncovered which appeared to control SVF. As there was no overlap between QTLs for these traits, the data suggests that different mechanisms regulate the development of GCD and SVF in response to attempted *Ptr* infection. All the identified QTL had small-to-medium effects and none of them exhibited epistatic interactions. The number of QTL uncovered suggests that there are a number of polymorphic loci impacting either GCD or SVF. This is in contrast to race-specific disease resistance where protection is routinely conveyed by one QTL of major effect.

A number of Arabidopsis mutants have been described that exhibit reduced NHR against the cowpea rust *U. vignae* (Mellersh and Heath, 2003), which are compromised in either SA biosynthesis or signalling. Furthermore, natural variation in SA has been reported, although the underlying genes have not yet been identified. The map position of QTL controlling natural variation in SA signalling, however, are not congruent with any of the identified QTL underpinning the expression of NHR against *Ptr*. This data supports the findings that Arabidopsis mutants defective in either SA synthesis or SA signalling do not support significantly increased growth of *Ptr*. We also checked the identity of genes in the uncovered QTLs for NHR against *Ptr* for the presence of defence-related coding sequences. In the case of GCD, the QTL on chromosome one contained multiple nucleotide binding site-leucine rich repeat (*NBS-LRR*) genes, which play a major role in pathogen recognition (Dangl and Jones, 2001). Interestingly, this region also contained a syntaxin (*At3g11820*) coding sequence.

This class of protein has recently been shown to be required for penetration resistance in Arabidopsis against the non-adapted pathogen *Bgh* (Collins *et al.*, 2003). Of particular significance to GCD may be the presence of a functional homologue of the vertebrate defending against apoptotic cell death (*DAD*) *1*, which is also located within the boundaries of this QTL (Gallois *et al.*, 1997). The second QTL identified for GCD, located on chromosome three, did not possess any known genes related to plant defence or cell death, suggesting the involvement of a novel locus at this position.

With regard to SVF, the QTL on chromosome five possessed multiple receptor-like kinase (*RLK*) genes. RLKs are emerging as key receptors for pathogen-associated molecular patterns (PAMPs). In this context, the RLKs encode receptors for both a peptide derived from bacterial flagellin and bacterial EF-Tu (Zipfel *et al.*, 2004; Zipfel *et al.*, 2006). This region also contains a number of *NBS-LRR* genes (Dangl and Jones, 2001), a negative regulator of SA-dependent gene expression, constitutive *PR* (*CPR*) *5* (Bowling *et al.*, 1997) and mildew resistance locus O (*MLO*) *10*, a member of a class of genes required for susceptibility towards powdery mildew pathogens (Consonni *et al.*, 2006). The other QTL on chromosome four associated with this trait also contains *NBS-LRR* and *RLK* genes, in addition to a gene encoding callose synthase, which plays an important role in plant-pathogen interactions (Nishimura *et al.*, 2003). The QTL on chromosome two associated with this trait, however, does not contain any previously described defence-related genes. Further studies will now be required to define more accurately the relevant regions of the genome possessing candidate genes. This can be accomplished by employing additional RILs and near-isogenic lines and by global gene expression profiling following *Ptr* challenge in accessions at opposite ends of the SVF range. The QTL uncovered here suggest the presence of multiple loci in Arabidopsis controlling NHR against *Ptr*. Identification of the polymorphisms underpinning these QTL would provide significant new insights into the expression of NHR.

Chapter 5

Stomatal Function relative to *Ptr* Invasion

Introduction

Unlike powdery mildew, leaf rust lacks the ability of direct penetration. Thus *Ptr* rely solely on stomatal openings to gain access into inner leaf tissues for its establishment and further development which ultimately results in sporulation. It has been previously proposed that narrowing stomatal apertures may limit rust penetration and be an advantageous strategy for plant defence (Lee *et al.*, 1999). In this context, a role for oligogalactronic acid and chitosan was demonstrated for reduction of stomatal aperture in tomato and *Commelina communis* (Lee *et al.*, 1999). Chitosan (β-1,4-linked glucosamine) is a deacylated derivative of chitin that is a component of many fungal cell walls (Bartnicki-Garcia, 2006). Chitin may act as a PAMP-like molecule and can be recognised by PRR (Nurnberger *et al.*, 2004). Chitosan also induces phytoalexin accumulation in the pea plants infected with *Fusarium solani* (Hadwiger and Beckman, 1980). With regard to bacterial pathogens, it is widely assumed that stomatal opening are passive entry ports. However, it was recently demonstrated that stomatal closure is part of the plant immune response (Melotto *et al.*, 2006). Stomatal closure against bacterial pathogen triggers a PAMP induced immunity system. Surprisingly, plant pathogenic bacteria have evolved specific virulence factors which cause stomatal reopening (Melotto *et al.*, 2006).

Stomatal aperture has been reported to be regulated through abscisic acid (ABA) or methyl jasmonate (MeJA) signalling, which are located upstream of ROI signalling (Murata *et al.*, 2001; Suhita *et al.*, 2004). MeJA also is involved in both plant development and immunity (Creelman and Mullet, 1997). The stomatal complex comprise a pair of guard cells, which determines a central pore. In guard

cells, ABA induces oxidative burst that in turn activates Ca^{2+} channels in the plasma membrane (Pei *et al.*, 2000). The elevated cytosol free Ca^{2+} causes activation of slow anion channels and inactivation of inward rectifying K^+ channels. Consequently, the K^+ efflux is followed by reduction of guard cell turgor and stomatal closure (Orozco-Cardenas and Ryan, 1999). The stomatal closure induced through MeJA is suppressed in the NADPH oxidase double mutant *atrbohDatrbohF* (Suhita *et al.*, 2004). In this chapter, I studied the influence on stomatal apertures in response to *Ptr* during the first 24 hrs of infection in Arabidopsis and wheat plants. Included in my observations were several Arabidopsis mutants defective in SA and ABA signalling pathways. The tested genotypes of wheat plants contained both resistant and susceptible cultivars.

Results

Stomatal Responses against *Ptr* in Arabidopsis

Recently it was demonstrated that perception of the bacterial pathogen *Pst*DC3000(*avrRpt2*) induces rapid stomatal closure in Arabidopsis, providing a barrier against infection (Melotto et al. 2006). As entry of *Ptr* into host tissues via stomatal penetration is a critical step towards successful host colonization, we investigated whether stomatal closure was triggered in Arabidopsis following attempted *Ptr* infection. *Pst*DC3000 triggered stomatal closure was SA and abscisic acid (ABA) dependent (Melotto *et al.*, 2006). We therefore examined *Ptr*-induced stomatal closure in the Arabidopsis lines *sid2* and *nahG* defective in SA accumulation and *aba3*, compromised in the biosynthesis of ABA (Xiong *et al.*, 2001; Bittner *et al.*, 2001). Experiments were conducted in randomised complete block (RCB) design with four replications. Plants were infected with a mixture of talcum powder and *Ptr* urediospores and subsequently kept in a dark dew chamber at 20° and 100% relative humidity. Mock treatment consisted of

talcum powder only. Leaves of 4 seedlings of each genotype were harvested at 0, 3, 6, 9, 12, 15, 18, 21 and 24 hours post inoculation (hpi). Collected leaves were immediately fixed in the FEA solution (formaldehyde 9%, ethylene 82% and acetic acid 9%) all v/v (volume/volume) ratio (Geisler and Sack, 2002) for 30 min. An area of 2-2 mm square from each leaf was selected randomly and the frequency of open / closed stomata scored by bright-filed microscopy. Any stomatal aperture less than 0.5 μm (Fig 5.1A) scored "closed stomata" as it was described previously (Melotto *et al.*, 2006).

An accumulation of hydrogen peroxide was observed where a *Ptr*-stoma challenge took place (Fig. 5.1B). Predominantly, the stomatal aperture of all Arabidopsis genotypes was significantly reduced when challenged with *Ptr*, for instance at 12 hpi only 24% of stomata were open (Fig. 5.2). In contrast, 88% of stomata were open in mock-inoculated control plants. However, all genotypes showed an increased frequency of open stomata (FOP) up to 3hpi. After 3hpi all genotypes except *aba-3* showed a decreased FOP in a similar fashion and the differences between infected and control plants began to be revealed (Fig 5.2A-E).

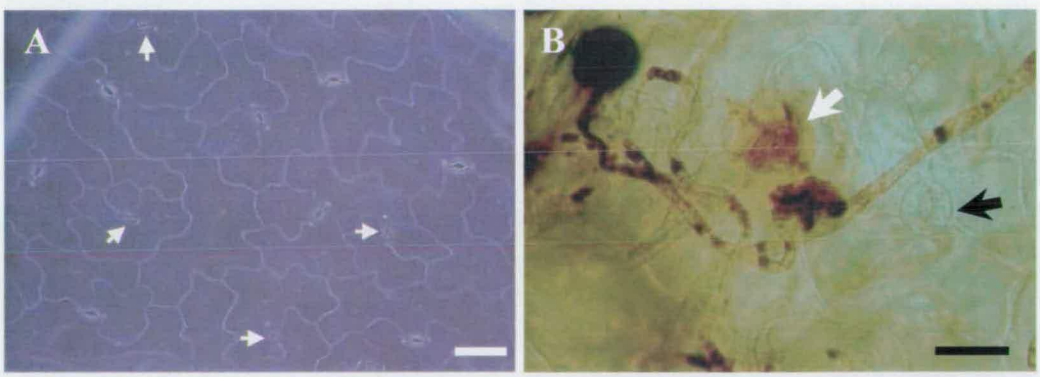


Figure 5.1. A) Light microscopy of the stoma aperture on Arabidopsis, arrow shows closed stoma, scale bar 25 μm; B) Reduction in stoma aperture upon *Ptr* interaction in Arabidopsis. DAB stain around closed stoma (white arrow) demonstrates presence of hydrogen peroxide accumulation. Subsequent *Ptr* attempted penetration of adjacent open stomata (black arrow) scale bar 25 μm.

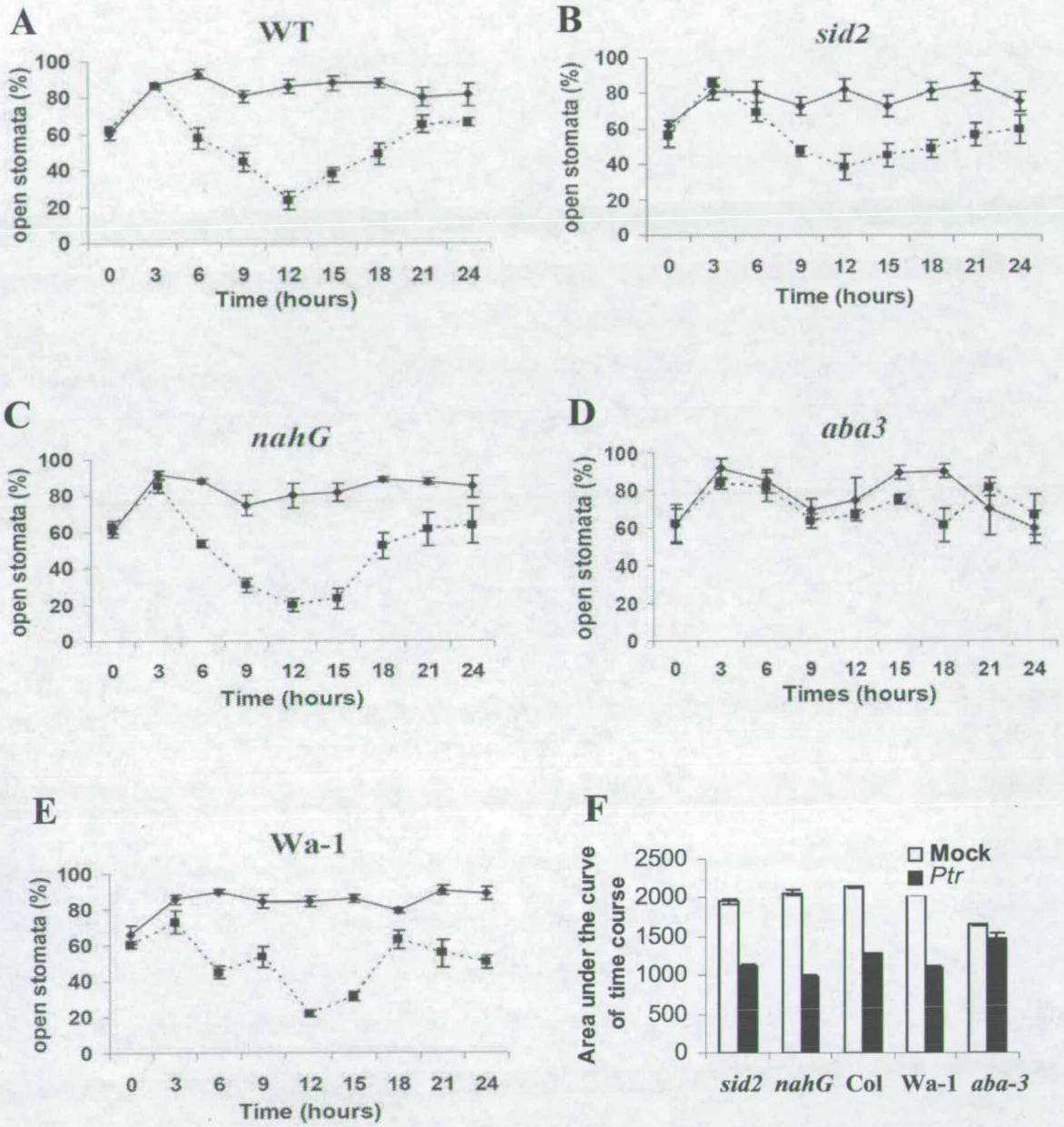


Figure 5.2. *Ptr* triggers stomatal closure in Arabidopsis. The given genotypes were challenged with *Ptr* and the percentage of open stomata determined over time; A-E) Diamonds intersected with an unbroken line and squares intersected with a broken line correspond to mock inoculated and *Ptr* inoculated values, respectively; F) Comparison of the overall stomatal closure during 24hpi indicates the given genotypes responded similarly to leaf rust, unlike *aba-3* .Error bars represent SE. These experiments were repeated twice with similar results.

While the *sid2* and *nahG* lines followed a similar trend to that of wild-type plants (Fig. 5.2B and C), *Ptr*-induced stomatal closure was abrogated in the *aba3* line (Fig. 5.2D). We also analyzed this defence response in the Wa-1 accession, which supports enhanced *Ptr* SVF. Stomatal closure in this line followed a similar trend to that of wild-type (Fig. 5.2E).

The trend of the FOP curve appeared similar among infected Arabidopsis plants but not in *aba-3*. The similarity of FOP trend among genotypes was confirmed when the overall FOP during the 24 hour period post infection showed a non-significant variation among of them (Fig. 5.2F). However, in the case of *aba-3* the FOP was not significantly changed when infected with *Ptr* and this mutant was significantly different from the other tested genotypes.

Stomatal Responses against *Ptr* in Wheat

Wheat leaf rust in all Arabidopsis genotypes triggered NHR. Therefore we were interested to investigate whether virulent *Ptr* is able to manipulate stomatal aperture in their host plant, wheat. In this context, we studied variation in the FOP in two resistance cultivars, Robin and Savanah. In addition, these resistant cultivars were compared with a susceptible (Vuka) cultivar. The experimental procedure applied here was similar to the one described in the last section. During the first 3 hours all genotypes in both infected and control treatments showed a striking increase in the FOP. Then, FOP decreased among infected genotypes (Fig 5.3 A-C). This was significantly different from their mock treated plants, suggesting host-*Ptr* sensing induces a reduction in stomatal aperture in the host plants regardless of genetic background. The variation in FOP was negligible for the two resistant cultivars while in case of the susceptible cultivar Vuka, the FOP began to increase at 15 hpi (Fig 5.3A), where it was significantly higher than control treatment.

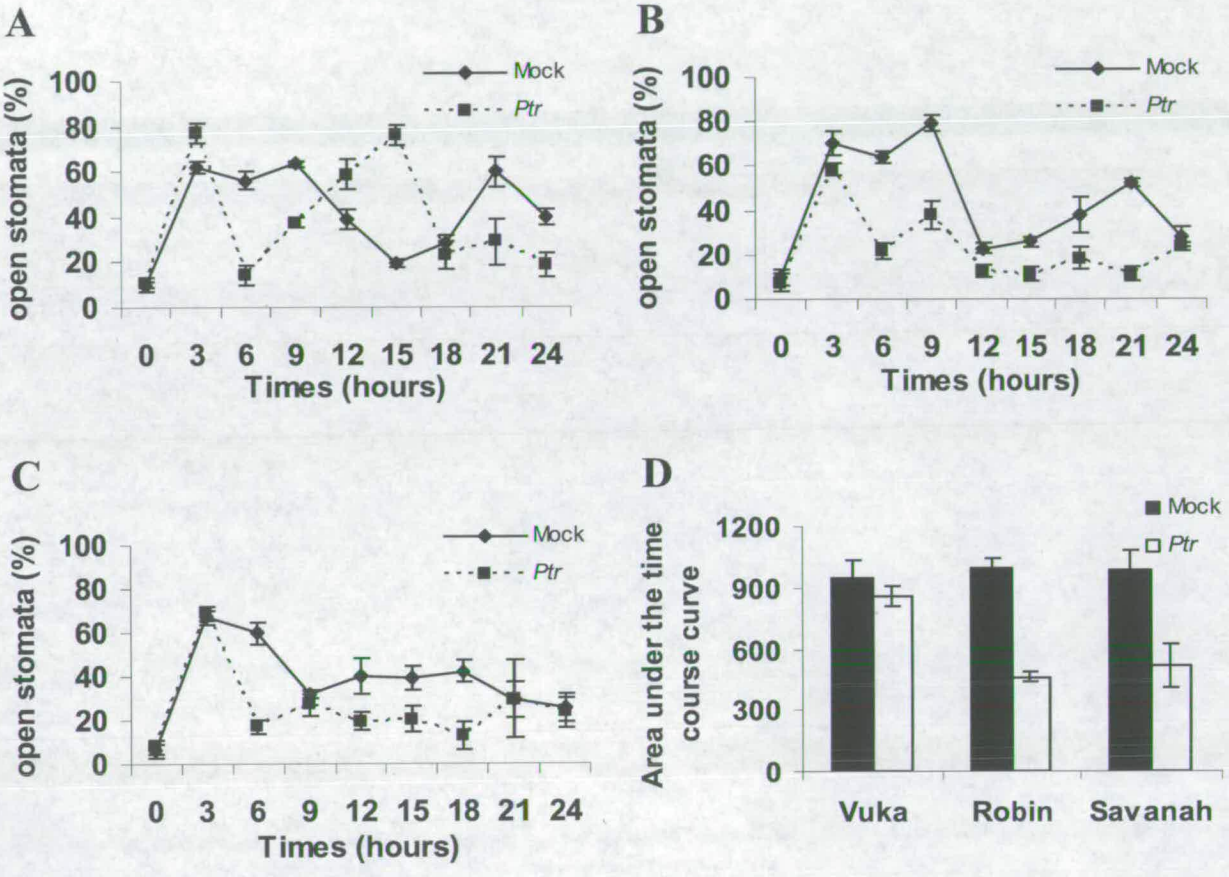


Figure 5.3. *Ptr* triggers stomatal closure in resistant wheat cultivars. The given genotypes were challenged with *Ptr* and the percentage of open stomata determined over time. A) Vuka, susceptible cultivar; B)Robin, resistant; C) Savanah, resistant; D) Comparison of the overall stomatal closure during 24hpi indicates theresistant cultivars responded similarly to leaf rust, while the overall changes in Vuka was not significantly different from mock. Error bars represent 95% confidence limits. These experiments were repeated twice with similar results.

The overall changes in the FOP for the 24 hour period of experiment showed that the frequency of open stomata in infected Vuka was not significantly lower than the mock treated plants. In contrast, infection of resistant cultivars resulted in a significantly FOP reduction (Fig 5.3 D).

Discussion

Stomatal pores are essential for water transpiration and gas exchange necessary for photosynthesis. However, they also provide potential portals within the epidermis for pathogen invasion. The current model suggests that a SA- or ABA-dependent signalling cascade regulates stomatal closure (Melotto *et al.,* 2006; Suhita *et al.,* 2004; Merlot *et al.,* 2007). In this context, several downstream signal transduction components were identified such as NO and hydrogen peroxide (Fan *et al.,* 2004). Generally, activation of plasma membrane H^+-ATPases, stimulates stomatal opening (Kinoshita and Shimazaki, 2001). The effect of ABA or SA on the basal H^+-ATPase activity remains to be revealed. Although, it was demonstrated that exogenous application of ABA triggers production of H_2O_2, which then causes a rise in cytosolic free Ca^{2+} and consequently enforces stomatal closure (Kwak *et al.,* 2003). Our data showed that stomatal closure was accompanied by an elevation of hydrogen peroxide in guard cells, suggesting regulation of stomatal closure is mediated through hydrogen peroxide. However, we have already shown in pervious chapter that the NADPH oxidase mutants did not support an enhanced frequency of SVF or further development of *Ptr*. Our data also suggests that *Ptr* triggers stomatal closure in an ABA-dependent fashion during the establishment of NHR against this pathogen. This situation is similar to the bacterial PAMP-induced stomatal closure, as neither flg22 nor LPS triggered stomatal closure in *aba3* mutant (Melotto *et al.,* 2006).

Our data suggested a reduction in stomatal aperture in both Arabidopsis and resistant cultivars of wheat. These observations imply that NHR and *R* gene-

mediated resistance might equally trigger stomatal closure in response to attempted *Ptr* infection. Moreover in our experiments, regardless of genetic background, a suppression of stomatal aperture was observed in all wheat genotypes between 3-6 hpi. These data may suggest involvement of PTI in NHR against *Ptr*. Here PTI might serve as an alarm system (Nurnberger *et al.*, 2004) for the presence of *Ptr*, which trigger stomatal closure. In this context, chitosan, a key structural component of fungi, promotes stomatal closure in tomato (Lee *et al.*, 1999). In agreement with our experiments, it was recently shown that bacterial PAMP molecules such as flg22, LPS and *E.coli* O157:H7 initiate stomatal closure in Arabidopsis and tomato (Melotto *et al.*, 2006). This PAMP-triggered stomatal closure may potentially proceed to downstream signalling events leading to the activation of the *R* gene mediated defence system (Li *et al.*, 2005). Interestingly, a reduction of stoma aperture was also obvious in the susceptible Vuka between 3 to 6 hpi, suggesting resistance to *Ptr* was not totally compromised in Vuka. However, a specific experiment needed to address role of PAMP in resistance against *Ptr*.

Unlike Arabidopsis, the larger size of the stomata in wheat allows a few appressorium to penetrate one stoma simultaneously and form sub-stomata vesicles (Hu and Rijkenberg, 1998), which increases the chance of penetration. Moreover, our data showed an invariable and early increase in stomatal openings, which might have been imposed by the conditions of growth room (Niks, 2002) within which we conducted our experiments. It is also notable that *Ptr* begins penetration of host plants and formation of sub-stomatal vesicles as early as 3 hours post infection (Hu and Rijkenberg, 1998), which overlaps with the time that an elevated FOP was observed in all wheat genotypes. Therefore, the increased size of stomata and the high FOP would collectively be advantageous for penetration of *Ptr* into sub-stomatal cavity. Nevertheless, our data suggested that both tested resistant wheat cultivars exhibited effective pre-haustorium resistance to *Ptr* whereas *Ptr* successfully initiated haustoria in a susceptible cultivar. On the other hand, an increased number of open stomata were observed in Vuka relative to the mock treatment between 6-15 hpi. These modifications suggested a role for

Ptr in manipulating stomatal aperture on host plants. In contrast to Vuka, longer period of stomatal closure were observed in resistant cultivars, which might be a consequence of *R* gene mediated resistance. As previously, the immune system through *R* gene-mediated resistance precludes pathogen development subsequent to sub-stomatal vesicle formation in resistant plants (Hu and Rijkenberg, 1998). Formation of haustorial structures in plant-pathogen interactions is under the control of specific-specific signals from the fungus which may include defence suppressors that are necessary in maintaining basic compatibility (Fernandez and Heath, 1991). In addition, besides nutrient uptake, haustoria have an important role in the suppression of plant defences (Mendgen *et al.,* 1996; Voegele and Mendgen, 2003). Therefore, reopening of the stomata in susceptible cultivars might be mediated by those haustoria that were initiated following early penetration of infection hyphae within 6 hpi. Similar interactions were shown previously with bacterial virulence factors, such as coronatine, were able to suppress early defences and reopen stomata in Arabidopsis (Melotto *et al.,* 2006). The consistently reduced stomatal aperture that was observed in both resistant wheat cultivars and non-host Arabidopsis might also imply the involvement of *R* gene mediated resistance, while environmental conditions were in favour of stomatal opening. As penetration of *Ptr* germling into the sub-stomatal cavity occurs in any case up to 6 hpi, therefore, it is more likely that development of the pathogen triggers *R* gene mediated resistance subsequently. Thus it can be hypothesised that following penetration, if pathogen effectors successfully overcome a plant's *R* gene mediated resistance, then successful suppression of stomatal closure would result. On the other hand, *Ptr* was not able to develop significantly on *aba-3* plants relative to wild-type or the other tested Arabidopsis mutants. Thus, even in the presence of a significant increase in open stomata conveyed by *aba-3*, NHR against *Ptr* in Arabidopsis was still effective.

Chapter 6

The *AS1* Nuclear Activity Modulates Plant-Microbe Interaction

Introduction

Besides studying non-host disease resistance of Arabidopsis to *Bgt* and *Ptr* pathogens, I collaborated in another project to characterize the role of a developmental selector in immune responses. Recently it was revealed in our lab that loss-of-function mutations in Arabidopsis *ASYMMETRIC LEAVES* (*AS*)*1* unexpectedly enhanced resistance levels to necrotrophic fungi (Nurmberg *et al.*, 2007). On the other hand, *as1* plants were compromised in non-host and basal disease resistance, specifically against hemi-biotrophic bacterial pathogens. In advance, however, I provide background information regarding *AS1* and its function in leaf development.

The Arabidopsis *AS1* and its orthologs *ROUGH SHEATH (RS)2* in maize and *PHANTASTICA (PHAN)* in *Antirrhinum* control the transition between indeterminate and determinate growth during leaf development (Byrne *et al.*, 2000; Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999; Waites *et al.*, 1998). These genes are conserved MYB orthologs (R2R3MYB) which negatively regulate class I knotted 1-like homeobox (*KNOX*) expression in leaves (Byrne *et al.*, 2000; Schneeberger *et al.*, 1998; Timmermans *et al.*, 1999; Waites *et al.*, 1998; Stracke *et al.*, 2001). Indeterminate apical growth is under control of *KNOX* gene and repression of *KNOX* genes activity results in determinate growth (Lincoln *et al.*, 1994; Nishimura *et al.*, 1999). In Arabidopsis, the *AS1*-dependent pathway for leaf development requires *AS2* (Xu *et al.*, 2003). A combination of *AS1* and *AS2* is required to inhibit expression of *KNOTTED1* (*KN1*)-like homeobox gene family members in leaves (Byrne *et al.*, 2000; Semiarti *et al.*, 2001). Loss of function of *AS2* or mis-expression of *KN1* results in similar lobbed

leaf shaped morphology relative to *as1* (Hay *et al.*, 2002; Xu *et al.*, 2003). However, in contrast to *as1* mutants, *as2* plants or an inducible *KN1* line did not express significantly enhanced resistance to *B. cinerea* (Nurmberg *et al.*, 2007).

Previously, screening a pool of activation tagged lines identified a line that exhibited strikingly reduced susceptibility to *Botrytis cinerea* compared to wild-type plants. The corresponding mutation was recessive and co-segregated with insensitivity to glufosinate herbicide encoded by the T-DNA. Thermal asymmetric interlaced (TAIL)-PCR was employed to amplify DNA sequences flanking the inserted T-DNA (Sessions *et al.*, 2002). This technique revealed that a T-DNA was inserted 521 bp upstream of an open reading frame encoding a MYB-related regulator (MYB91), termed *AS1*. This T-DNA insertion prevented *AS1* transcription accumulation and is presumed to result in a null mutation. Resistance to *B. cinerea* was associated with lobed leaves (Nurmberg, 2006). The new mutation was confirmed as an allele of *AS1* in crosses to the classical mutation *as1-1* (Redei and HIRONO, 1964) and was therefore named *as1-4*. Furthermore, *as1-1* also conferred heightened resistance against *B. cinerea*, confirming the pleiotropic roles of *AS1* in pathogen resistance and plant development (Nurmberg *et al.*, 2007).

Lack of *AS1* function also confers increased resistance against *A. brassicicola*, a necrotroph pathogen (Nurmberg, 2006). In addition, *as1* plants exhibited significantly enhanced disease susceptibility against *Pst*DC3000. Interestingly, resistance to the non-host bacterial pathogen *P. fluorescens* 2-79 was also compromised in *as1* plants (Nurmberg, 2006). These observations collectively suggest an important role for *AS1* in plant-microbe interactions. Due to marked resistance of *as1* plants to *B. cinerea*, I will provide a brief introduction on grey mould disease, its control and a comparison between signalling pathways between biotroph and necrotroph pathogens.

Grey Mould

The grey mould, *B. cinerea* Pers. causes an important disease in over 200 plant species including crops, vegetables, flowers, small fruits, and other fruit trees. The importance of this disease is specifically highlighted in production of indoor vegetables such as tomato, eggplant and pepper (Agrios, 1997). Due to its broad spectrum of hosts, *Botrytis* is the most common necrotroph and imperfect fungus causing fruit and general diseases on plants. Disease symptoms can be observed on infected plants as blossom blights and fruit rots, damping-off, stem cankers or rots, leaf spots, and tuber, corm, bulb, and root rots (Agrios, 1997). Unlike biotrophic pathogens, necrotrophs kill host cells as soon as they invade a plant as a method to nutrient uptake. These types of pathogens are able to remain active on dead tissues (Williamson *et al.*, 1995).

Despite recent advances in crop protection, control of grey mould is still mainly dependent on fungicides such as imidazoles and dicarboximides. However, the efficiency of protection is seriously affected by the appearance of resistant strains (Baraldi *et al.*, 2002). Due to the rare appearance of the sexual form of *B.cinerea* in nature, it is unlikely that sexual reproduction is the main source of variation (Faretra *et al.*, 1988). However, the regular occurrence of aneuploidy and mutations are thought to be the major sources of genetic variability in *B. cinerea* (Buttner *et al.*, 1994).

Among hemi-biotrophic pathogens, *Pst*DC3000 the causative agent of bacterial speck is a common disease on tomato. This Gram-negative bacterium colonizes in the apoplast (Alfano and Collmer, 1996). Biotrophic and hemi-biotrophic pathogens trigger SA-mediated signalling pathways whereas resistance to necrotrophs are mediated through JA-signalling pathways. Some of the resistance responses in plants toward biotrophic pathogens such as the induction of reactive oxygen species (ROS) facilitate penetration of necrotrophic pathogens (Deighton *et al.*, 1999). With regard to penetration, cell wall degrading enzymes (CWDE) and ROS are important components of *Botrytis* pathogenicity compared to the

involvement of appressorium structures, which is mainly employed by biotrophic fungi (Deighton *et al.*, 1999; Viterbo *et al.*, 1993).

Here, I investigated the regulatory role of *AS1* in response to host and non-host pathogens in Arabidopsis and *Nicotiana sylvestris*. The aim of the research was to analyse the existence of epistasis relations of *as1* with any of the main genes mutated in the JA signalling pathway. I also studied whether the function of the *AS1* orthologs are conserved in *N. sylvestris*. The role of an *AS1* homolog from a primitive monocot plant species was also studied in the transgenic Arabidopsis. In addition I studied the localization of AS1 in response to a bacterial pathogen.

Results

AS1 function is conserved in *Nicotiana sylvestris*

The function of *AS1* and its orthologes was conserved in repressing *KN1*-like genes in leaves. We therefore determined if the functions of *AS1* in plant-pathogen interactions were also conserved between Arabidopsis and *Nicotiana sylvestris*. Arabidopsis *as1* plants showed a significant susceptibility to virulent *Pst*DC3000, *PstDC3000*(a*vrB*), *Pst*(*avrRps4*) and *P. fluorescens 2-79* compared to wild-type (Nurmberg, 2006). To investigate *AS1* function further, we evaluated the resistance of an *N. sylvestris* line which constitutively expresses antisense *NsPHAN*. Antisense *NsPHAN* leaves show ectopic expression of *NTH20*, a *KNOX* gene, and consequently misregulation of *KNOX* gene expression resulted in a series of developmental abnormalities. *N. sylvestris* plants with silenced *NsPHAN* are more susceptible to *Pst*DC3000 and *P. fluorescens 2-79* whereas they showed reduced lesion development in response to *B. cinerea* relative to wild-type. Similar results were obtained in *Antirrhinum* plants carrying a mutation in *PHANTASTICA* (Nurmberg *et al.*, 2007).

This antisense tobacco plants depleted in *NsPHAN* RNA were also infected with *Erwinia amylovora* (*Eam*), a non-host bacterial pathogen. Inoculation was carried out at a cell density of 10^8cfu/ml suspended in 10 mM $MgCl_2$. In the absence of *PHAN* function, resistance of *N. sylvestris* was significantly compromised against *Eam* (Fig 6.1A). This data is in line with the previous observations. Therefore, the role of *AS1* in pathogen interactions seems to be conserved in species with a divergence time of ~125 million years.

Further assessment was carried out to monitor the HR reaction in antisense *NsPHAN* and wild type *N. sylvestris* plants. An electro conductivity assay was used to quantify HR. Both genotypes were infected with the nonhost pathogen *Eam* at a cell density of 10^8 cfu/ml. Plants from both genotypes were also mock infected with 10 mM $MgCl_2$. Leaves from each plant were detached, weighed and placed in a Petri dish containing 10 ml of double distilled water. Then the amount of ion leakage was measured from infiltrated leaves at five time points up to 48 hours post infection. Measurements of fluids were done through the conductivity of fluid released from sample leaves. Faster HR is associated with faster release of leaf ions. At six hours post infection, ion release accelerated in both the wild type and the antisense lines. However, this pattern remained similar in both genotypes. Control and infected plants were significantly different. However, both genotypes remained similar throughout the experiment following challenge with *Eam* (Fig 6.1B). These results suggest that *PHAN* is not involved in regulating HR development in response to *Eam*.

Epistasis analysis of *as1*

Resistance to necrotrophic fungal pathogens typically correlates with induction of JA/ET-dependent genes (Thomma *et al.,* 1998). It was shown previously, in *as1* mutants induction of *PLANT DEFENSIN* (*PDF*) *1.2*, *PATHOGENESIS-RELATED* (*PR*) *3* and *PR4* genes were accelerated in response to *B. cinerea* (Nurmberg *et al.,* 2007). On the other hand, accumulation of *PR1* transcripts, a

key marker for the expression of salicylic acid (SA)-dependent disease resistance (Uknes *et al.,* 1992), and also SA synthesis, were not altered in *as1* mutants relative to Col-0 when inoculated with *Pst*DC3000 (Nurmberg *et al.,* 2007).

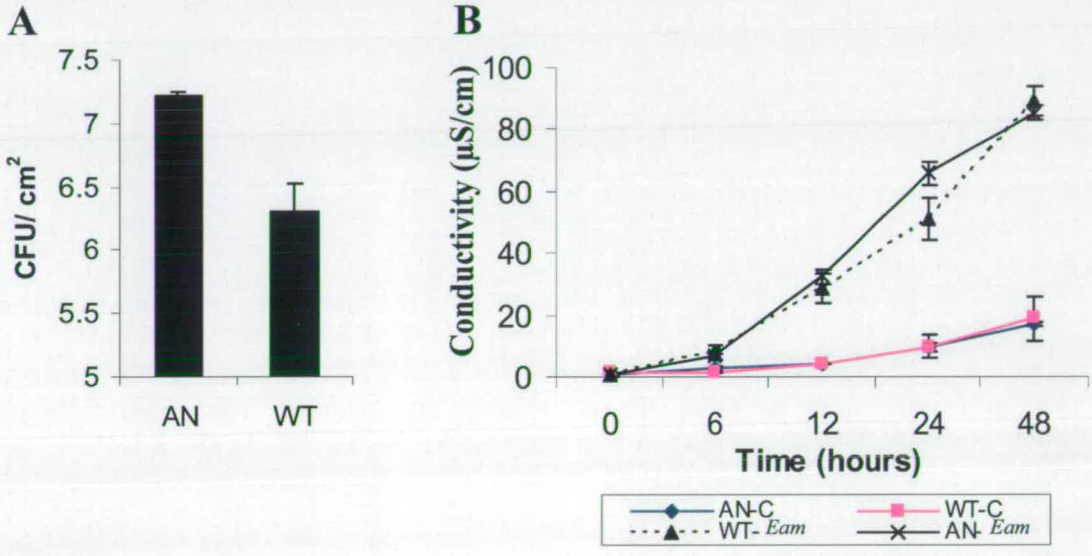


Fig 6.1. Response of *N. sylvestris* antisense line expressing *PHAN* antisense RNA to *E. amylovora*, A) Rate of *Eam* growth in antisense *PHAN* (AN) relative to wild-type (WT); B) AN-C: antisense of *PHAN* control treatment, WT-C: wild type control, AN-*Eam*: antisense line infected, WT-*Eam*: wild type infected. Inoculation for test of pathogenecity and HR carried out with a bacterial concentration of 10^8 and 10^6 CFU/ml, respectively. Experiments were repeated twice with similar results. Bars are 95% confidence intervals.

The widely reported antagonistic relationship between SA and JA (Schenk *et al.*, 2000; Glazebrook *et al.*, 2003; Spoel *et al.*, 2003), raised the hypothesis that any deficiencies in JA signalling may stimulate activation of SA-dependent signalling and enhances the level of resistance to *Pst*DC3000 in *as1* plants. Thus, to identify possible defence components that may be required for *as1*-mediated resistance, we created a series of *as1* double mutants to uncover whether JA/ET signalling modifies the regulatory role of *as1* in the interaction with *Pst*DC3000. We therefore examined the responses of *as1coi1*, *as1ein2*, *as1AOS* antisense and *as1*(a*AOS*), lines of Arabidopsis against virulent *Pst*DC3000. *as1*(a*AOS*) is a line expressing an antisense transcript of the *allene oxide synthase* (*AOS*) gene, which is required for JA biosynthesis (Bell *et al.*, 1995). *COI1* is required for JA-mediated signalling (Feys *et al.*, 1994), while *EIN2* is a key component of the ET signalling pathway (Guzman and Ecker, 1990). The mutant lines *coi1* and *ein2* are recessive whereas the antisense *AOS* transgene is dominant.

The *as1ein2* double mutant was selected on MS plates supplemented with the ET precursor aminocarboxilic acid (ACC) at a final concentration of 10 μM (Thomma *et al.*, 1999). The F2 seedlings were grown in the dark at 4°C in the presence of ACC for 7 days. Those lines did not display the triple response (due to a mutation in *EIN2*) were transferred onto soil and double mutants selected by the morphological phenotype of *as1*. The *as1*(a*AOS*) plants were identified in F2 by the morphological phenotype of *as1* and male sterility conferred by the *AOS* antisense transgene, which were then rescued by exogenous application of Me-JA at buds of conditionally sterile plants. Selection of *as1coi1* double mutants was made on MS plates containing 10 μM MeJA (Feys *et al.*, 1994). Root and shoot development of the double mutants was not inhibited under this condition. The desired seedlings were then removed from the plates after 10 days and planted in soil. Double mutants were identified through the morphological phenotype of *as1*.

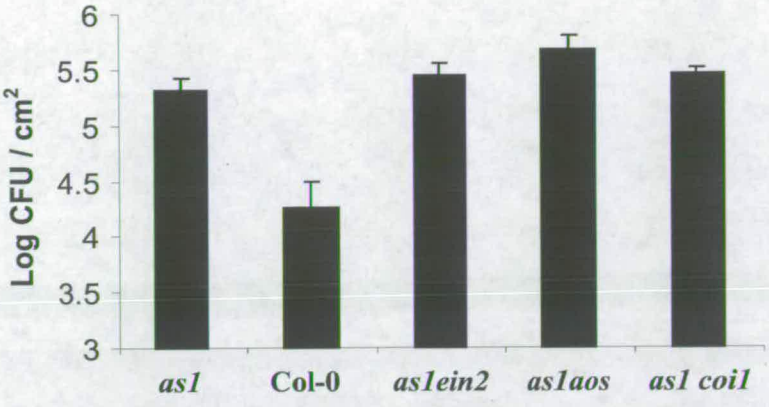


Figure 6.2. Responses of *as1* double mutants to *Pst*DC3000. Growth of *Pst*DC3000 scored at 4dpi and compared to wild type Col-0. Experiments were repeated twice with similar results. Bars represent 95% confidence intervals.

In this context, *as1ein2*, *as1coi1* and *as1*(a*AOS*) double mutant genotypes were inoculated with *Pst*DC3000 at a concentration of 10^6 CFU/ml and assessed at 4 dpi. The enhanced susceptibility of *as1* mutants remained unchanged as the *as1* plants and its double mutant lines expressed a similar level of susceptibility compared to Col-0 (Fig 6.2). These results suggest an independent role for *AS1* in the regulation of bacterial interactions in Arabidopsis, which is not associated with resistance to necrotrophic pathogens. One possibility could be the involvement of *AS1* in repressing production of a host-susceptibility factor required specifically for bacterial colonisation.

SkARP1 complements leaf morphology in *as1* plants but not *Pst*DC3000 susceptibility

AS1 has been the subject of extensive research due its key role in leaf development. *KNOX* and *AS1* genes are expressed in mutually exclusive domains and their interactions in the shoot apex balances indeterminate and determinate growth (Lincoln *et al.*, 1994; Nishimura *et al.*, 1999). A homologue of *AS1* from a primitive monocot plant species, *Selaginella kraussiana* is able to complement

the lobed leaf phenotype in the dicot Arabidopsis *as1* mutant (Harrison *et al.*, 2005). This has been shown by transformation of *as1* mutants with *SkARP1* under the control of a constitutive promoter. Furthermore, *SkARP1* has the ability to down-regulate expression of *KNOX* gene in the Arabidopsis leaf and to restore the leaf morphology of *as1* plants to wild-type shape. Thus, it was concluded that *SkARP1* is functionally equivalent to *AS1* in Arabidopsis with respect to leaf development (Harrison *et al.*, 2005).

Here, we tested the response of the *as1 35S:SkAPR1* line to *Pst*DC3000 and *B. cinerea*. Interestingly, our experiments showed that *SkARP1* did not complement the function of AS1 in response to *Pst*DC3000. Both *as1* and *as1 35S:SkAPR1* plants expressed enhanced susceptibility relative to Col-0 wild-type, at 3 dpi when infected with 10^6 CFU/ml of *Pst*DC3000 (Fig 6.3A-D). Likewise, both *as1* and *as1 35S:SkAPR1* plants exhibited a higher level of pathogen growth compared to wild-type (Fig 6.3E). Interestingly, *as1 35S:SkAPR1* and Col-0 showed similar lesion size and infection phenotypes when infected with *B. cinerea* (data not presented). This implies that *SkARP1* complements *as1* in the response against *B. cinerea*.

To investigate this observation further, we compared the amino acid (aa) structures of the AS1 and SkARP1. The AS1 protein contains 367 aa, whereas SkARP1 has 404 aa. A protein blast comparison for these proteins showed that they share similar aa sequences particularly within their first 108 aa sequences (Fig 6.4). Motif scanning tools from TAIR and EXPASy websites detected two MYB domains within the shared region. The enhanced susceptibility of *as1* plants to *Pst*DC3000 was not complemented by *SkARP1*. In contrast, *SkARP1* complemented the resistance of *as1* plants towards *B. cinerea*. These results have two important ramifications. First it suggests that AS1 has separate function in the response of Arabidopsis to *B. cinerea* and *Pst*DC3000. Moreover, it implies that the role of AS1 in response to *Pst*DC3000 is more ancient than its role in response to *B. cinerea*.

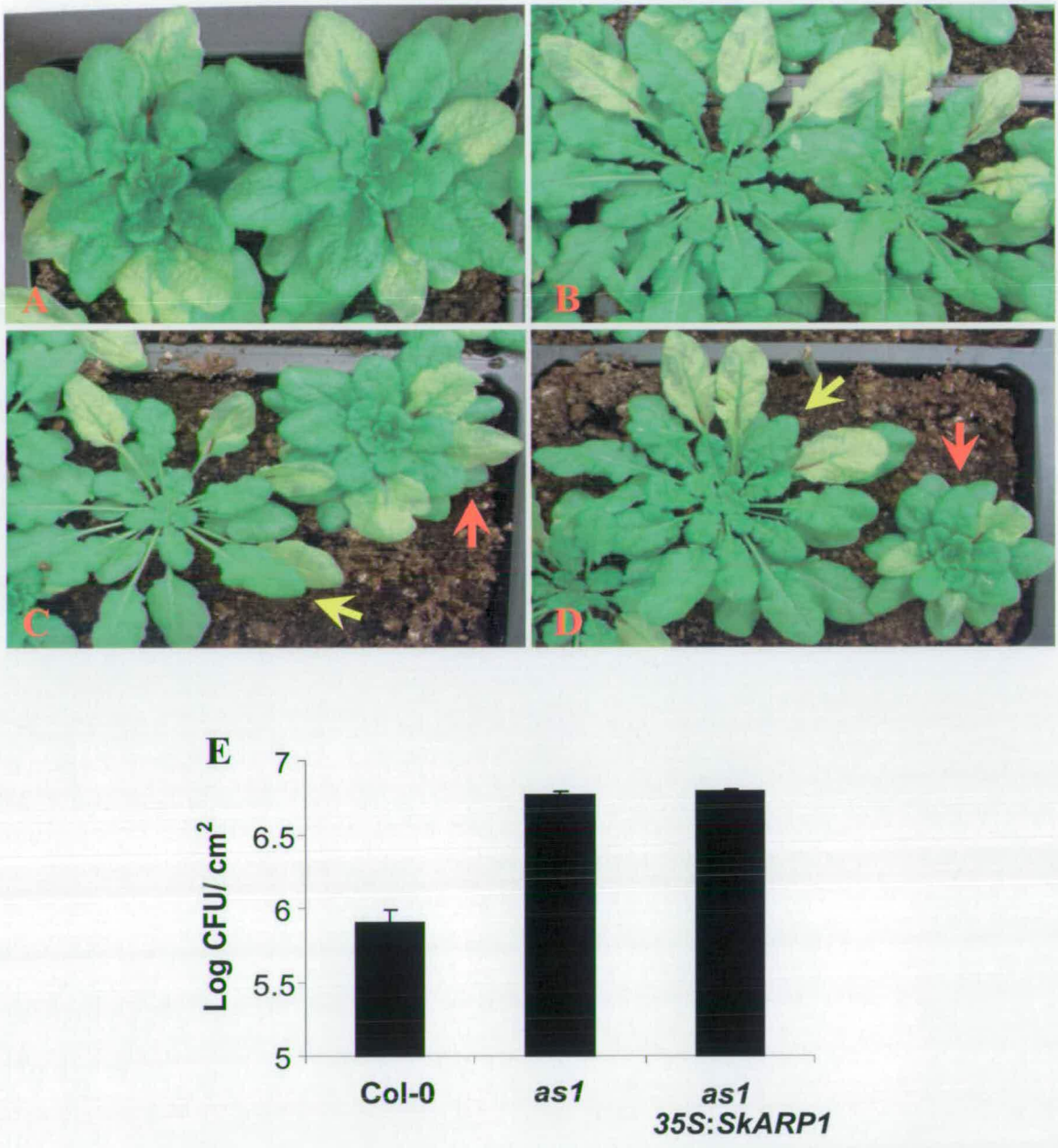


Figure 6.3. *as1* and *as1 35S:SkARP1* lines inoculated with *Pst*DC3000. A) *as1* plants; B) *as1* mutants complemented with *SkARP1*; C,D) yellow arrows points to *as1 35S:SkAPR1* plants and red arrows to *as1* plants. Pale yellow leaves were infected with 10^6 CFU/ml of *Pst*DC3000 suspension. Images were taken at 3 dpi; E) Both *as1* and *as1 35S:SkAPR1* plants exhibit similar increased *Pst*DC3000 growth compared to wild-type Col-0. Plants were infected with 10^6 CFU/ml of virulent *Pst*DC3000 and samples collected at 3dpi. Experiments were carried out twice with similar results. Bars represent 95% confidence intervals.

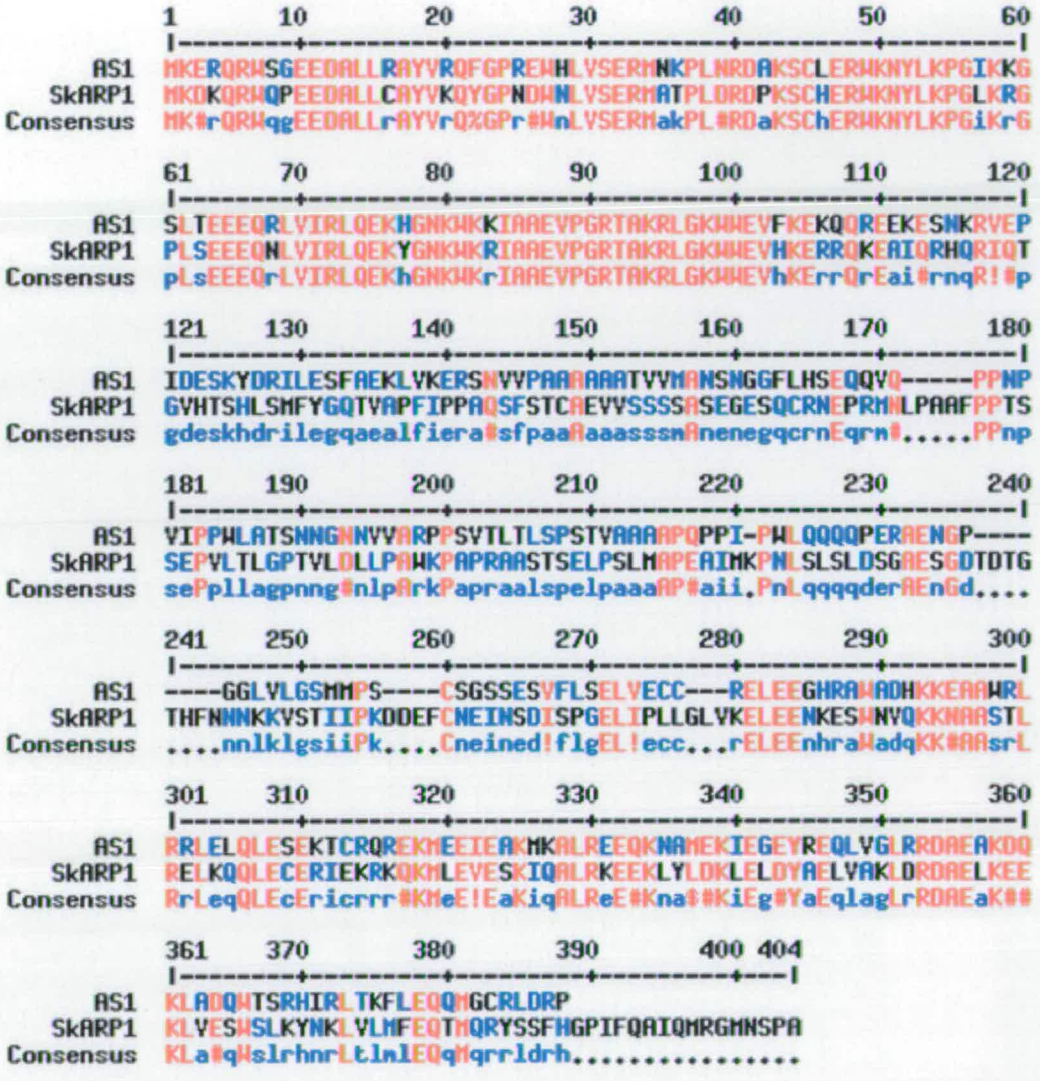


Figure 6.4. Amino acid structure of AS1 and SkARP1. High similarity exists in the first 108 aa sequences. Red colour shows high consensus value (90%), blue is low consensus value (50%) and neutral presented in black. Consensus symbols are as follow: "! is anyone of IV; $ is anyone of LM; % is anyone of FY; # is anyone of NDQEBZ"

Localization of AS1 in response to *Pst*DC3000

Recent evidence suggests a nuclear localization for AS1 (Theodoris *et al.*, 2003). However, possible localization changes in response to pathogens remained to be examined. In this context, we investigated the sub-cellular localization of AS1 by employing the Green Fluorescence Protein (GFP) as a marker (Theodoris *et al.*, 2003). A transgene expressing a RS2::GFP fusion complemented both the developmental and defence-related phenotypes of *as1* mutants, revealing that RS2 is functionally equivalent to AS1 (Theodoris *et al.*, 2003; Nurmberg *et al.*, 2007).

GFP is an 11-stranded ß-barrel protein that has the ability of absorbing ultraviolet light and then releasing it as low energy green light. GFP can be used as a fluorescent probe that is commonly used to instantaneously detect rapid biochemical changes within a cell. Thus an experiment was carried out by employing Arabidopsis plants transformed with *RS2::GFP* in order to study AS1 localization in response to *Pst*DC3000 (Theodoris *et al.*, 2003).

Accordingly, using confocal laser scanning microscopy, we studied the RS2::GFP fusion protein in 4 week old plants. Plants were inoculated with 10^6 CFU/ml of *Pst*DC3000 and leaves where collected at different time points for confocal imaging. The cell nuclei were stained with DAPI, distinguishing them with a blue colour. DAPI fluorescence was excited by bluediode laser at 405 nm and emissions detected between 480 to 500 nm whereas GFP fluorescence was excited at 488 nm, and emissions were detected between 505 to 530 nm.

RS2 has been demonstrated to be a nuclear protein, as it was exclusively localised in nuclei (Theodoris *et al.*, 2003). In the leaf epidermis, we observed a constitutive accumulation of RS2::GFP in the plants nuclei at zero time points (Fig 6.5A-C). Similarly, at 24 hpi RS2::GFP observed in nucleus only. However, relocation of RS2 in the nuclei was clear (Fig 6.5D-F). In some cases a reduction in GFP brightness was observed, which might be attributed to protein turnover. Although, due to lack of mock treatment with $MgCl_2$ in our experiments, it

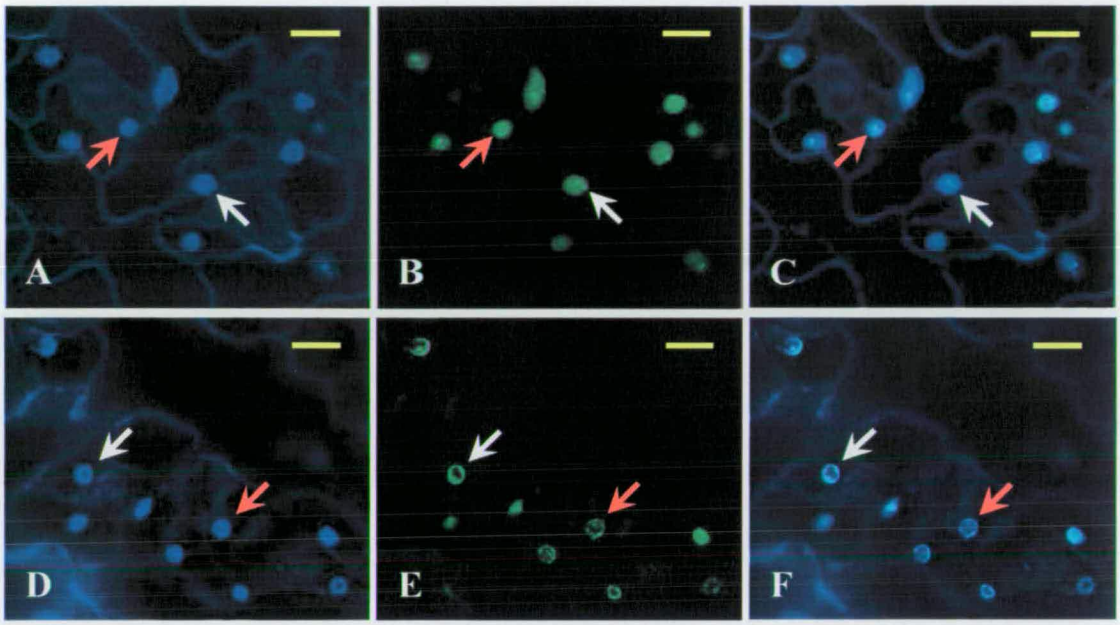


Figure 6.5. Localization of AS1 protein in response to *Pst* DC3000 in the epidermis. Confocal image of the leaf epidermis cells at zero hpi (A-C) and 24 hpi (D-F); DAPI used as a nucleus marker (A and D); green fluorescent signal (B and E) and overlay images (C-F) are shown. Arrows show nucleus, same colours in each row points to the same nucleus. The scale bar corresponds to 10μm.

remained unclear whether the changes in localization were consequence of pathogen infection or wounding due to the subsequent infiltration of bacteria.

We also investigated the intensity of GFP fluorescence in the challenged plants with *Pst*DC3000 in the mesophyll layer. At this stage, we added chlorophyll fluorescence as an extra control marker to the background. The autofluorescence of chlorophyll can be excited by an argon ion laser at 488 nm with emissions detected between 650-700 nm in the orange-red region of the spectrum. GFP signals were monitored at 0, 5, 10 and 24 hours post infection. At all time points, RS2::GFP was localized in the nucleus, which was similar to the localization of this protein in epidermal cells.

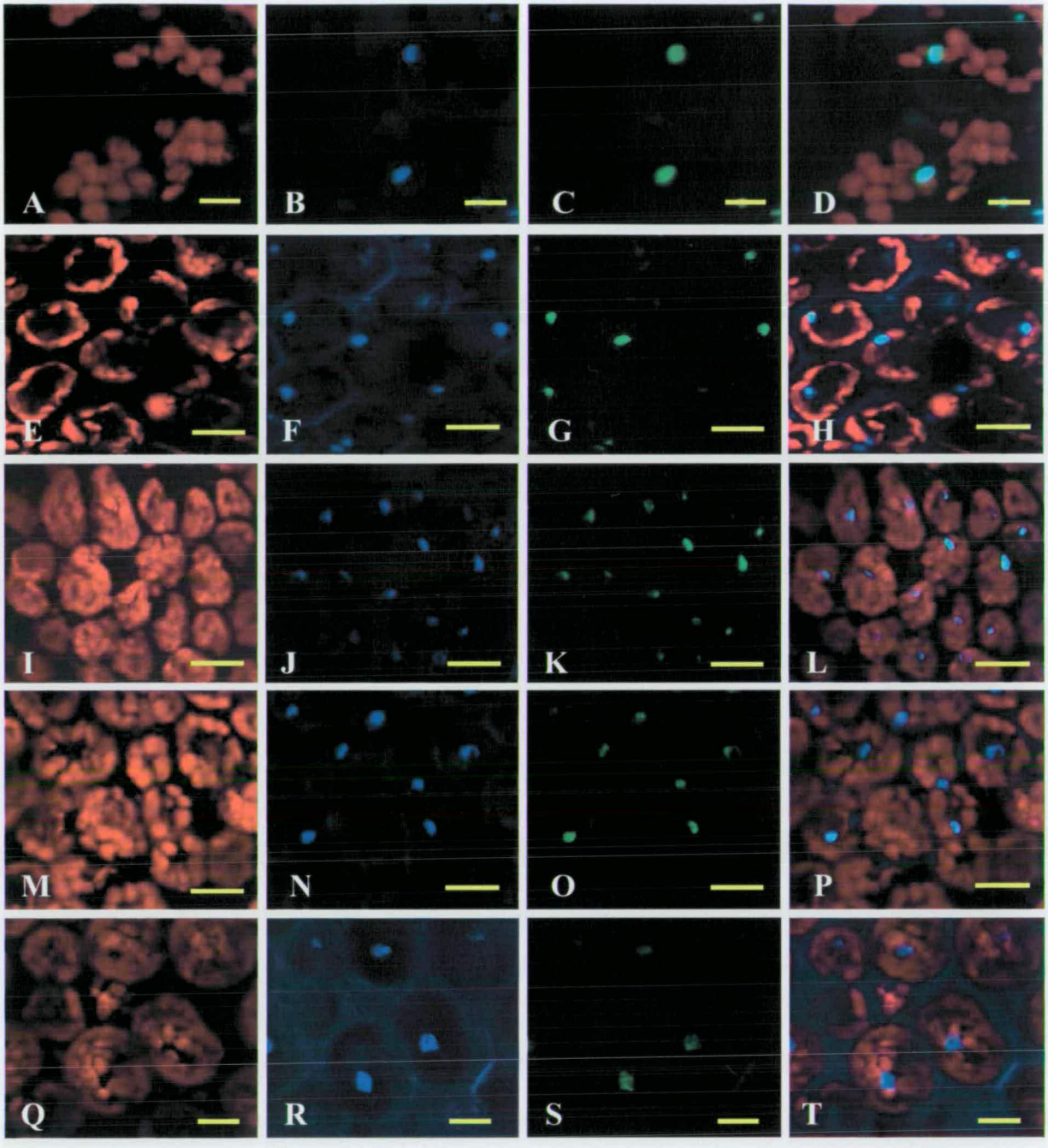


Figure 6.6. Localization of AS1 protein in response to *Pst*DC3000 in mesophyll cells. Confocal image of the leaf mesophyll cells at zero hpi (A-C); 5 hpi (E-H);10 hpi (I-L) and 24 hpi (M-T); chlorophyll red autofluorescence used as a chloroplast marker (A,E,I,M,Q); DAPI used as a nucleus marker (B,F,J,N,R); green fluorescent signal (C,G,K,O,S) and merged images (D,H,L,P,T) are shown. The scale bar corresponds to 10, 15, 20, 15 and 10 μm from top row, respectively.

However, the intensity of GFP signals varied during the experiment. At up to 5 hpi signals did not change significantly (Fig 6.6A-H). However, at 10 hpi signals were noticeably decreased (Fig 6.6I-L). This reduced level of AS1 was maintained until 24 hpi (Fig 6.6M-T).

Reduction in RS2::GFP may also be due to protein turnover, as rapid protein turnover ensures that regulatory proteins are degraded so that the plant cell can respond to changing conditions (Rojo *et al.,* 2003). However, the photobleaching of RS2::GFP might be another possibility. Therefore specific experiments should be designed in order to quantify the variation of AS1 in response to *Pst*DC3000. Our data, in general, suggest a nucleolus localized regulatory role for AS1 under stressful conditions.

Discussion

AS1 is a MYB transcription factor and belongs to the *R2R3* family. Members of this gene family are involved in many different plant functions including the plant immune system (Stracke *et al.*, 2001). The negative correlation between *AS1* and homeobox genes i.e. *KN1* and *KN2* determines plants growth habit (Byrne *et al.*, 2000). Moreover, AS1 interacts physically with AS2 to regulate the adaxial-abaxial polarity of leaves (Xu *et al.*, 2003; Xu *et al.*, 2006). Recently an important role was discovered for *AS1* in plant immunity (Nurmberg *et al.,* 2007). This was in addition to its well studied role in leaf development, which is conserved between plant species with divergence time of over 200 million years (Theodoris *et al.,* 2003; Mchale and Koning, 2004). The role of *AS1* in the regulation of the plant immunity system is independent of *AS2* or *KNAT* genes function (Nurmberg *et al.,* 2007) suggesting *AS1* might interact with different proteins in order to regulate resistance responses compared to the role of AS1 in development. A similar autonomous function for *AS1* and *AS2* was also demonstrated in flower development (Xu *et al.,* 2003).

Mutation in *AS1*, enhances resistance to *B. cinerea* but it is associated with significantly suppression of resistance to different *Pseudomonas syringae* pathovars including avirulent strains (Nurmberg, 2006). The involvement of *AS1* in plant-microbe interactions was conserved in three evolutionary diverse plant species (Nurmberg *et al.,* 2007). Likewise, a *N. sylvestris* line with silenced *NsPHAN* expressed an increased level of susceptibility to *Eam*, a non-host pathogen of tobacco plants suggesting a conserved function for *AS1* in relation to bacterial responses.

Transformation of Arabidopsis *as1* lines with an *ARP* gene from *Selaginella kraussiana* (*SkARP1*) restored wild-type leaf shape and suppressed resistance against *B. cinerea* relative to *as1* plants. However, this gene did not alter the level of susceptibility to *Pst*DC3000 compared to *as1* plants. Analysis of aa structures of both proteins revealed high similarities between the protein in their first 108 amino acids, which contain two probable MYB domains. The *ARP1* gene isolated from *S. kraussiana* has sufficient homology with Arabidopsis *as1* to complement leaf morphology. However, due to the genetic distances between Arabidopsis and *S. kraussiana*, this monocot *ARP* gene failed to interact with the resistance counterparts in Arabidopsis in order to mediate resistance. Therefore, these data suggest that *SkARP1* can uncouple the role of *AS1* in plant-microbial interactions against *Pst*DC3000 and *B. cinerea*. Taken together, AS1 function in resistance against *B. cinerea* seems to be more ancient than the role of *AS1* in response to *Pst*DC3000.

A mutation in *as1* was associated with an accelerated induction of JA/ET-dependent genes (Nurmberg *et al.,* 2007). In addition, in response to *B. cinerea*, *as1ein2* double mutants and particularly *as1coi1* plants exhibited increased lesion development relative to the *as1* line and were compromised in JA/ET-dependent gene expression, suggesting *as1*-mediated resistance against *B. cinerea* requires ET and JA signalling (Nurmberg *et al.,* 2007). On the other hand, loss of JA/ET did not alter the level of susceptibility/resistance of *as1* to *Pst*DC3000. This data suggests a specialised role for *AS1* in the regulation of resistance to this bacterial

pathogen. Therefore, *AS1* appears to promote resistance to bacterial pathogens through a previously uncharacterised mechanism. In one possible scenario, *AS1* could be a negative regulator of the proteins required for either biosynthesis or transport of a metabolite required specifically for bacterial colonisation. Loss of *POWDERY MILDEW RESISTANCE* (*PMR*)*6* function results in resistance against *E. chichoracearum* in the absence of SA-, JA- or ET-dependent defence responses (Vogel *et al.,* 2002). Thus, *PMR6* has also been proposed to function as a host susceptibility factor. These situations may occur during the arms-race of co-evolution, as pathogens enforce selection criteria to subvert plant metabolites in terms of nutrient acquisition and pathogenesis factor (Bergelson *et al.,* 2001).

Mutation of *SYMPTOMS TO MULTIPLE AVR GENOTYPE* (*SMA*) *4*, enhances resistance of Arabidopsis to *B. cinerea* but compromises resistance of the mutant plants to *Pst*DC3000(a*vrB*) and *Pst*DC3000(*avrRpt2*). Unlike *AS1*, however, resistance of *sma4* to *B. cinerea* is independent of *ein2* or *coi1* (Tang *et al.*, 2007). *SMA4* encodes a member of the long chain acyl-CoA synthases family, LACS2, which functions in cutin synthesis (Tang *et al.*, 2007). In another example, mutation in *BOTRYTIS SUSCEPTIBLE* (*BOS*) *1* resulted in enhanced susceptibility to *B. cinerea* and *A. brassicicola.* However, it was associated with increased disease symptoms of *Pst*DC3000 and *H. parasitica* but did not increase pathogen growth (Mengiste *et al.*, 2003). *BOS1* in a similar fashion to *AS1* interacts with the JA signalling pathway and encodes a R2R3MYB transcription factor protein (Mengiste *et al.*, 2003). Again comparable to *as1*, despite the enhanced symptoms of *Pst*DC3000 observed in *bos1* plants, expression of *PR-1* was not altered (Mengiste *et al.*, 2003).

Employing RS2::GFP, demonstrated that AS1 is localized in the nucleus. The intensity of GFP signals and also localization of RS2 within nuclei varied during the 24 hours after *Pst*DC3000 inoculation. These changes might be attributed as a regulatory function for AS1 in response to either *Pst*DC3000 or wounding. However, these preliminary data can be helpful in designing more accurate experiments in this regard.

Chapter 7

General discussion

The plant immune system tackles the majority of pathogens through NHR. Understanding this type of resistance still remains a challenge. However, recent studies have identified several genes and components that are involved in NHR (Ellis, 2006; Nurnberger and Lipka, 2005). A non-host disease interaction in plants is usually accompanied by diverse defence responses, which may imply there are great incompatibilities and a complex genetic basis behind NHR (Ellis, 2006; Heath, 1996). Therefore, NHR might orchestrate different components of the immune system i.e. at early stages inducing PAMP-triggered immunity (PTI) and at later stages involving effector-triggered immunity (ETI), which is equal to *R* gene mediated resistance and/or basal resistance (Jones and Dangl, 2006; Lipka *et al.,* 2005).

Mutant screening strategy is one of the popular methods, which has been successfully employed to uncover several genes involved in NHR (Collins *et al.,* 2003; Hostettmann and Marston, 1995; Lipka *et al.,* 2005; Lu *et al.,* 2001; Stein *et al.,* 2006). On the other hand, the co-evolutionary arms-race model (Dawkins and Krebs, 1979) provide a suitable ground for variability between geographical accessions, as natural selection normally applies lenient and low selection pressures. In this context, the exploitation of natural variation proved to be a powerful tool for gene discovery (Borevitz and Chory, 2004; Shindo *et al.,* 2007). Particularly, the development of well characterised mapping populations such as RILs (Lister and Dean, 1993), unlocked a great potential for identifying gene(s) underpinning complicated characteristics

NHR is essentially effective at Pre-penetration stage

Here, I screened 79 Arabidopsis accessions against *Ptr* and *Bgt* for NHR. Invariably, all accessions exhibited strong NHR without any visible disease symptoms or plant resistance phenotypes. However, variations were observed on different accessions in terms of restricted penetration. Although both *Bgt* and *Ptr* are biotrophic fungal pathogens, they have demonstrated considerably different infection strategies (Mendgen *et al.*, 1996). Pre-haustorial resistance was the distinct feature of NHR in our experiments where more than 90% of pathogen hyphae ceased to penetrate. This feature distinguished NHR from *R* gene mediated resistance in our experiments. As *R* gene mediated resistance occurs predominantly at the post-haustorial stage and is associated with the HR (Hammond-Kosack and Jones, 1997; Heath, 1996). On the other hand, the remaining penetration sites were effectively defended by callose deposition or programmed cell death mostly as soon as the penetration peg initiated a connection. This uniformity in the defence response among accessions supports the hypothesis that plants evolved a detection system for multiple and highly conserved PAMPs by pattern recognition receptors (PRRs) (Nurnberger *et al.*, 2004). This early recognition system (PTI) is a component of the plant immune system, which contributes to NHR (Ausubel, 2005; Nurnberger *et al.*, 2004). Although, PTI mediates a weak immunity system (Jones and Dangl, 2006), it evidently plays an important role in activation of downstream defence signal transduction (Zipfel *et al.*, 2006; Mishina and Zeier, 2007). PTI triggers activation of conserved MAPK signalling cascades, induction of defence related genes, production of antimicrobial compounds, accumulation of ROS and NO, callose deposition and stomatal closure (Asai *et al.*, 2002; Fellbrich *et al.*, 2002; Melotto *et al.*, 2006; Zeidler *et al.*, 2004; Ramonell *et al.*, 2005; Navarro *et al.*, 2004; Nurnberger *et al.*, 1997; Kaku *et al.*, 2006). In the *Bgt*-Arabidopsis pathosystem, our data might suggest that PRRs activated NHR. However, a number of minor natural mutations provided a platform for an increased number of pre-mature penetrations on some of the accessions such as Wc-1. Similar examples were observed in Arabidopsis where mutations in *PEN1, PEN2* or

PEN3 resulted in an increased penetration rate of *Bgh* without further development of the pathogen (Collins *et al.,* 2003; Lipka *et al.,* 2005; Stein *et al.,* 2006). A higher rate of penetration in Wc-1 was correlated with an increased rate of HR. Although, the extent of similarities between HR reactions in host and non-host resistance remained to be understood, this reaction could be attributed as a consequence of a flux of non-functional or non-compatible AVR proteins secreted by *Bgt* into the Arabidopsis epidermis and mediated through a PAMP induced pathway (Ellis, 2006). Collectively, despite impaired pre-penetration NHR in Wc-1, an effective post-penetration NHR might combat the non-host pathogen, *Bgt.*

In another attempt to compromise NHR in Arabidopsis against *Bgt*, cytochalasin E, an inhibitor of actin polymerisation, was applied onto *eds1* mutants preceding inoculation, which resulted in partial susceptibility to *Bgt* (Yun *et al.*, 2003). Similar treatment on the Wc-1 accession only increased the frequency of penetration by around 4% and did not support the growth of secondary hyphae. The higher frequencies of penetration can be assigned to impaired rearrangement of the cytoskeleton, as the increased penetration rate was also observed in wild-type. On the other hand, further development of *Bgt* growth on *eds1* plants might be due to *R* gene activity, as *EDS1* is involved in both TIR-NBS-LRR signalling and basal resistance (Aarts *et al.,* 1998; Parker *et al.,* 1996). Nevertheless, NHR was able to abolish further growth of *Bgt* on *eds1* plants effectively (Yun *et al.*, 2003). Therefore, *R* genes may be important elements in NHR and they could be involved in PAMP-induced signalling events (Jones and Dangl, 2006; Zipfel *et al.,* 2004). Therefore, at the first stage it might be necessary for a non-adapted pathogen e.g. *Bgt*, to overcome or bypass PTI for infection (Chisholm *et al.*, 2006). In a compatible interaction, virulence factors in pathogenic bacteria were able to shut-down the PAMP-induced signalling events successfully (He *et al.*, 2006). However, this reasoning was generated from similar situations and the role of PTI in *Bgt*-Arabidopsis pathosystem remains to be addressed in specifically designed experiments.

Ptr* triggers more complicated NHR than *Bgt

Rust infection involves access into the inner layers of leaf tissue before initiation of haustoria inside the mesophyll cells. This process requires exploiting topographical and chemical cues to locate stomata (Collins *et al.*, 2001). Our data demonstrated that on a non-host plant, germ tubes of *Ptr* are mainly unable to identify a penetration pore. On average, only 12% of *Ptr* germ tubes were able to detect Arabidopsis stomata. However, *Ptr* challenge still resulted in the activation of inducible defence responses including the oxidative and nitrosative burst, the engagement of salicylic acid (SA)-, jasmonic acid (JA)- and reactive oxygen intermediate (ROI)-dependent gene expression, SA accumulation and camalexin biosynthesis and stomatal functions. Taken together, these multi-faceted responses point toward the complexity of NHR in Arabidopsis against *Ptr*, which might involve many genes with minor impact. Lack of variation among well characterised defence-response mutants against *Ptr* may also be attributed to the complexity of this interaction as any of these pathways alone do not play a crucial role in this pathosystem. In contrast, mutation in *ATGSNOR1*, *EDS1*, *PAD4* and *SAG101* reduced the level of NHR against *Bgt* and *Bgh* in Arabidopsis (Feechan *et al.*, 2005; Lipka *et al.*, 2005; Yun *et al.*, 2003).

NHR in *Ptr*-Arabidopsis pathosystem stimulates JA-dependent genes whereas the expression of NHR against *Bgt* did not involve JA signalling (Yun *et al.*, 2003) therefore, highlighting potential mechanistic differences between the expression of defence responses in Arabidopsis against these two non-adapted fungal pathogens. While SA and JA signalling have been reported to be mutually antagonistic (Gupta *et al.*, 2000; Felton *et al.*, 1999), JA and SA function synergistically to drive the expression of *PDF1.2* (Mur *et al.*, 2006) and other genes (Schenk *et al.*, 2003). Furthermore, over-expression of the coiled-coil-nucleotide binding site-leucine rich repeat (*CC-NBS-LRR*) gene *ADR1*, resulted in the expression of both *PDF1.2* and SA-dependent genes (Grant *et al.*, 2003) and JA responses have recently been shown to underpin systemic acquired resistance (SAR) (Truman *et al.*, 2007). Thus, the relationship between JA and SA

signalling in the context of attempted *Ptr* infection is likely to be highly complex and probably time and concentration dependent.

On the other hand, *Ptr* triggers stomatal closure in an ABA-dependent fashion during the establishment of NHR against *Ptr*. This response might contribute to NHR by diminishing the number of sites available for *Ptr* entry into the sub-stomatal cavity. Recognition of one or more PAMPs associated with *Ptr* by the host may trigger this response. In this context, chitosan, a key structural component of fungi, promotes stomatal closure in tomato (Lee *et al.,* 1999). Furthermore, the bacterial PAMPs flg22 and LPS both initiate stomatal closure in Arabidopsis and tomato (Melotto *et al.,* 2006).

Natural variation may uncover genetics of NHR against *Ptr*

Screening accessions of Arabidopsis revealed Wa-1 exhibited the highest frequency in formation of *Ptr* sub-stomatal vesicles among the other lines tested and counter-intuitively key defence responses in this accession were both stronger and more rapid compared to Col reference plants. In this context, our data support the notion that the enhanced growth of *Ptr* in the sub-stomatal cavities in Wa-1 plants triggered more powerful defence responses in this line. This may reflect a quantitative reduction in the effectiveness of non-inducible defence mechanisms in Wa-1 plants, deployed preceding host-cell penetration, compared to wild-type. Thus, increased *Ptr* development in the Wa-1 line triggers a more powerful inducible defence response compared to that observed in wild-type plants. A similar phenomenon is observed for the *pen* mutant series (Collins *et al.,* 2003; Lipka *et al.,* 2005; Stein *et al.,* 2006) which have compromised penetration resistance but exhibit increased activation of inducible defence responses.

Exploring the genetic control of NHR against *Ptr* defined five QTLs. Two QTLs govern GCD, and three unrelated QTLs appeared to control SVF. The QTLs for GCD and SVF could conceivably function in the same genetic pathway: for example, supporting the identification of host stomata by *Ptr*. An increase in the number of stomata identified by *Ptr* may then result in both enhanced GCD and SVF. However, there was no overlap between QTLs for these traits. Furthermore, negative correlation between GCD and SVF suggesting an inverse relationship. Collectively, these findings therefore suggest that the QTLs for GCD and SVF may regulate different genetic pathways.

The QTLs for GCD contained multiple *NBS-LRR* genes (Dangl and Jones, 2001) and of particular significance to GCD, presence of a functional homologue of the vertebrate defending against apoptotic cell death (*DAD*) *1* (Gallois *et al.,* 1997). On the other hand, the QTLs for SVF possessed multiple receptor-like kinase (*RLK*) genes, which are emerging as key receptors for PAMPs. In addition, several *NBS-LRR* genes (Dangl and Jones, 2001), a negative regulator of SA dependent gene expression, constitutive *PR* (*CPR*) *5* (Bowling *et al.,* 1997) and mildew resistance locus O (*MLO*) *10*, a member of a class of genes required for susceptibility towards powdery mildew pathogens (Consonni *et al.,* 2006) were detected in QTLs for SVF. The QTL uncovered here suggest the presence of multiple loci in Arabidopsis controlling NHR against *Ptr*. Further studies will now be required to define more accurately the relevant regions of the genome possessing candidate genes. This can be accomplished by employing additional RILs and near-isogenic lines and by global gene expression profiling following *Ptr* challenge. Moreover, combining genome-wide expression profiling with genetic linkage analysis could help to elucidate the molecular basis of complex traits (Gibson and Weir, 2005). In a recent approach, expression quantitative trait loci (eQTLs), gene expressions scanned from microarray transcription profiles are used as a quantitative trait, which further are compared to a genetic map to find markers associated to the candidate gene (s) (Zhang *et al.,* 2007). These eQTLs are particularly informative if phenotypic QTLs (pQTLs) were identified, since the eQTLs uncover the potential downstream genes of the pQTL (DeCook *et al.,*

2006). Variation in genomic sequence within or close to the regulated gene results in identification of *cis*-acting eQTLs, which are the candidate genes for pQTLs (Zhang *et al.,* 2007). On the other hand, *trans*-regulated eQTLs reflect differences in remotely regulated gene expression. The presence of *trans*-acting eQTLs points toward the possibility of co-ordinated regulation of many genes by a single 'master regulator'. These master regulators are probably key control points in gene networks and any variation in these regulators result in complex phenotypes (Yvert *et al.,* 2003).

Non-defence related genes modulate plant-microbial interactions

MYB transcription factors are involved in many different plant functions including the plant immune system (Stracke *et al.*, 2001). Among them, negative regulation of homeobox genes i.e. *KN1* and *KN2* by *AS1* determines plants growth habit (Byrne *et al.*, 2000). In addition, *AS1* independent of *AS2* or *KNAT* genes play a role in the regulation of plant immunity system (Nurmberg *et al.,* 2007). Mutation in *AS1* simultaneously increases resistance to *B. cinerea* and *A. brassicicola* as well as susceptibility to virulent and avirulent strains of *Pst*DC3000 and *P. fluorescens*, which is a non-host pathogen of Arabidopsis (Nurmberg *et al.,* 2007). However, the level of resistance in *as1* did not alter in response to *E. cichoracearum* (*Ec*) and *Bgt* relative to wild-type plants. These data suggest an impaired basal, *R* gene-mediated and also non-host resistance in *as1* plants specifically to bacterial pathogens (Nurmberg *et al.,* 2007). *AS1* function in plant development and immunity is conserved between plant species with a divergence time of ~ 125 million years (Mchale and Koning, 2004; Nurmberg *et al.,* 2007; Theodoris *et al.,* 2003; Waites *et al.,* 1998). In this context, we demonstrated that the level of resistance in *N. sylvestris* plants with silenced *NsPHAN* was significantly decreased to *Eam*, a non-host pathogen of tobacco plants. This is another indication of the conserved function of *AS1* in relation to bacterial pathogens. Interestingly, an *AS1* ortholog gene from

Selaginella kraussiana (*SkARP1*) unfolded the role of *AS1* in plant-microbial interactions against *Pst*DC3000 and *B. cinerea*. The *AS1* function relative to *B. cinerea* was associated with wild-type leaf morphology in the transformed Arabidopsis plants with *SkARP1*. These findings also suggest *as1*-mediated resistance to *B. cinerea* has an ancient genetic origin.

Resistance of *as1* plants to necrotrophic pathogens might be explained through the JA signalling pathway, as an accelerated expression of JA-dependent defence genes were observed in *as1* plants following *B. cinerea* challenge. Furthermore, *as1* double mutants with either *ein2* (Guzman and Ecker, 1990) or *coi1* (Feys *et al.*, 1994), which convey insensitivity towards ET or JA respectively, exhibited a compromised resistance to *B. cinerea* (Nurmberg *et al.*, 2007). However, the suppressed resistance against bacterial pathogens in the light of *PR-1* expression and SA accumulation is counter-intuitive. On the other hand, the level of resistance against *Pst*DC3000 in the *as1* double mutants, *as1coi1* and *as1ein2* and *as1*(a*AOS*) remained unchanged relative to *as1* plants. These data collectively suggest *AS1* might modulate resistance to bacteria through a previously uncharacterised mechanism, and it is unlikely that *AS1* represent a direct component of the SA-dependent signalling pathway. It is notable that resistance to biotrophic pathogens is typically dependent on the SA signalling pathway (Klessig and Malamy, 1994; Hammond-Kosack and Jones, 1997). Therefore, it might be assumed that *AS1* plays distinct roles in the establishment of disease resistance, such as serving as a negative regulator of susceptibility factors for bacterial colonisation. An example for a susceptibility factor is *PMR6*. Resistance of *pmr6* plants was increased against *E. chichoracearum* in the absence of SA-, JA- or ET-dependent defence responses (Vogel *et al.*, 2002). In another case, *sam4* Arabidopsis plants exhibit enhanced resistance to *B. cinerea* but increased susceptibility to *Pst*DC3000(a*vrB*) and *Pst*DC3000(*avrRpt2*) (Tang *et al.*, 2007). Unlike *AS1*, however, resistance of *sma4* to *B. cinerea* is independent of *ein2* or *coi1* (Tang *et al.*, 2007). SMA4 functions in cutin synthesis (Tang *et al.*, 2007). Taken together, our data and the impact of *SAM4* or *PMR6* on resistance, suggests immunity to certain pathogens may be induced independently of known defence

signalling pathways. Plants species inadvertently synthesize potential susceptibility factors as part of their integral biology (Stachel *et al.,* 1986; Vogel *et al.,* 2002; Vogel *et al.,* 2004). Therefore according to the co-evolutionary arms-race model (Dawkins and Krebs, 1979), it can be anticipated that pathogens evolve mechanisms to exploit these weak points for their pathogenesis.

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